PhD thesis

The pathogenic significance of IL-23/Th17 axis, regulatory T cells, and platelet activating-factor (PAF) in the psoriatic phenotype of K5.hTGF-β1 transgenic mice

submitted by

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Dedicated to my parents

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1. SUMMARY

The IL-23/Th17 and regulatory T cell (Treg) axis were studied in the pathogenesis of skin disease in K5.hTGF-β1 transgenic mice, exhibiting a skin phenotype and cytokine abnormalities with strong similarities to human psoriasis. An impaired function of CD4+CD25+ Tregs and increased cytokine levels of the IL-23/Th17 pathway were responsible for the psoriatic phenotype in this mouse model. These conclusions were mainly deduced from the interference with the pathogenesis of disease by (1) psoralen+UVA (PUVA) photochemotherapy, (2) anti-IL-17 antibody treatment, and (3) platelet-activating-factor (PAF) injection or blockade of its receptor.

(1) K5.hTGF-β1 transgenic mice were treated with PUVA, a standard dermatological therapy. It suppressed the IL-23/Th17 pathway, Th1 milieu, as well as transcription factors STAT3 and orphan nuclear receptor RORgammat. PUVA induced the Th2 pathway and IL-10 producing CD4+CD25+Foxp3+Tregs (iTregs) with disease-suppressive activity that was abolished by anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) antibody treatment. These findings were paralleled by macroscopic and microscopic clearance of the diseased murine skin, which indicated that both iTregs involving CTLA4 signaling and inhibition of the IL-23/Th17 axis are central for the therapeutic action of PUVA.

(2) Anti-IL-17 antibody was used to neutralize the bioactivity of IL-17. Injecting anti-IL-17 mAB intraperitoneously into K5.hTGF-β1 transgenic mice diminished the psoriatic phenotype of the mice. Treatment with anti-IL-17 mAb did not show any effect on FoxP3 and IL-10 mRNA expression in the skin of K5.hTGF-β1 transgenic mice suggesting independent involvement of Treg and Th17 axis in psoriasis.

(3) Injecting PAF into the skin of transgenic mice led to inflammation and accelerated the manifestation of the psoriatic phenotype by a local effect. In contrast, injecting mice intraperitoneously with PAF receptor antagonist PCA-4248 lowered the PAF level (most likely by depressing an autocrine loop). In addition PCA-4248 treatment also lowered neutrophil, CD68+ cell (monocyte/macrophage) and CD3+T cell accumulation in the skin and blocked progression of the psoriasis-like phenotype. This effect of PAF blockade was similar to that of PUVA and paralleled by a decrease of abnormally elevated mRNA and/or protein levels of Th17-related cytokines IL-17A, IL-17F, IL-23, IL-12A, and IL-6, and the transcription factor STAT3. In contrast, PCA-4248 treatment upregulated the mRNA levels of COX2 and IL-10 in the dorsal skin and release of IL-10 in the serum and skin.

These findings imply that specifically interfering with IL-23/Th17, Tregs and/or PAF may be an option to induce cytokine pathways with disease suppressive activity and develop novel anti-psoriatic therapeutic strategies in particular for hyper-inflammatory disease variants and associated systemic comorbidities.

Pathogenetische Bedeutung der IL-23/Th17-Achse, der regulatorischen T-Zellen und des Blutplättchen-aktivierenden Faktor (PAF) beim psoriatischen Phänotyp von K5.hTGF-β1 transgenen Mäusen

ZUSAMMENFASSUNG

IL-23/Th17 und regulatorische T-Zellen (Treg) wurden in der Pathogenese der Hautveränderungen bei K5.hTGF-β1 transgenen Mäusen studiert, die einen Phänotyp und Zytokinabnormalitäten aufweisen, die denen von Patienten mit Psoriasis ähnlich sind. Die Untersuchungen ergaben eine verminderte Funktion von CD4+CD25+Foxp3+ Treg und erhöhte Zytokinspiegel der IL-23/Th17-Kette, welche für die psoriatischen Hautveränderungen im untersuchten Modell verantwortlich waren. Diese Schlussfolgerungen ließen sich von der Beeinflussung der Krankheitspathogenese mit (1) Psoralen+UVA (PUVA)-Photochemotherapie, 2) anti-IL-17-Antikörper-Therapie und 3) Blutplättchen-aktivierendem Faktor (platelet-activating factor, PAF) oder PAF-Rezeptorblockade ableiten.

(1) K5.hTGF-β1 transgene Mäusen wurden mit PUVA, einer dermatologischen Standardtherapie behandelt. PUVA supprimierte die IL-23/Th17-Achse, das Th1-Milieu, die Transkriptionsfaktoren STAT3 und Orphan Nuclear Receptor RORgammat. PUVA induzierte die Th2-Achse und IL-10-produzierende CD4+CD25+Foxp3+ Treg (iTreg) mit krankheitssupprimierender Aktivität, welche durch eine Behandlung mit anti-zytotoxischem T-Lymphozyten-Antigen-4 (CTLA4) aufgehoben wurde. Diese Ergebnisse ergaben sichparallel zur makroskopischen und mikroskopischen Rückbildung der erkrankten psoriatischen Haut, was darauf hinwies, dass sowohl iTreg über CTLA-Signalling, als auch die Hemmung der IL-23/Th17-Achse von zentraler Bedeutung für die Wirksamkeit von PUVA sind.

(2) Eine Behandlung mit anti-IL-17-Antikörper diente zur Neutralisierung der Bioaktivität von IL-17. Die intraperitoneale Injektion von IL-17-Antikörper reduzierte die Ausprägung des psoriatischen Phänotyps. Die Behandlung mit IL-17-Antikörper beeinflusste die Expression von FoxP3 und IL-10 mRNA in der Haut der K5.hTGF-β1 transgenen Mäuse allerdings nicht, was auf eine unabhängige Rolle von Treg und Th17 bei Psoriasis hindeutet.

(3) Die Injektion von PAF in die Haut von transgenen Mäusen führte zur Entzündung und beschleunigte das Auftreten des psoriatischen Phänotyps durch eine lokale Wirkung. Im Gegensatz dazu verminderte die intraperitoneale Verabreichung des

PAF-Rezeptor-Antagonisten PCA-4248 die PAF-Spiegel in der Haut (höchstwahrscheinlich über Suppression einer autokrinen Schleife). Die Behandlung mit PCA-4248 verminderte auch die Anhäufung von neutrophilen Granulozyten, CD68+ Zellen (Monozyten/Makrophagen) und CD3+T-Zellen in der Haut und hemmte die Progression des psoriatischen Phänotpys. Die Wirkung der PAF-Blockade war der einer PUVA-Behandlung sehr ähnlich und verminderte die abnormal erhöhten mRNA- und/oder Proteinspiegel der TH17-Zytokine IL-17A, IL-17F, IL-23, IL-12A und IL-6 sowie des Transkriptionsfaktors STAT3. Im Gegensatz dazu erhöhte die Behandlung mit PCA-4248 die mRNA-Spiegel von COX2 und IL-10 in der Haut und die Freisetzung von IL-10 in das Serum und in die Haut.

Diese Ergebnisse implizieren, dass eine Interaktion mit der IL-23/Th17-Achse, Treg und/oder PAF die Möglichkeit eröffnet Zytokine mit krankheitssupprimierender Aktivität zu induzieren und somit neuartige anti-psoriatische Therapiestrategien insbesondere für hochentzündliche Krankheitsvarianten und Komorbiditäten zu entwickeln.

2. INTRODUCTION

2.1. Psoriasis

Psoriasis is a chronic inflammatory skin disease affecting approximately 3 to 5 % population of US and Europe (Christophers, 2001). Psoriasis is an organ-specific autoimmune disease that is triggered by the result of interplay among environmental, immune and genetic factors. The general characteristics of psoriasis includes a) hyperplastic epidermis (i.e acanthosis) with hyperkeratosis and/or parakerotosis, b) increased inflammatory cell infiltrates including T cells, mononuclear cells (macrophages and monocytes), neutrophils, dendritic cells and mast cells in the dermis of the psoriatic skin, and c) increased blood vessel formation (i.e angiogenesis) in the dermis of the psoriatic skin (Lowes *et al.*, 2007a; Nestle *et al.*, 2009a; Schon and Boehncke, 2005a).

2.1.1. Genetic factors

Genomewide linkage analysis has identified nine chromosomal loci that are associated with psoriasis. These loci are classified as psoriasis susceptibility 1 to 9 (PSORS1-PSORS9) (Bowcock and Krueger, 2005). PSORS1 is located within the major histocompatibility complex (MHC) on chromosome 6p and accounts as a main heritability factor of the disease (Nestle *et al.*, 2009a). Furthermore, studies have also reported that IL-23 receptor (IL23R) and the untranslated region of the IL-12B (p40) gene are being associated with the risk of psoriatic susceptibility (Capon *et al.*, 2007; Cargill *et al.*, 2007). In addition, CDKAL1, ZNF313, PTPN22, IL-4/IL-13 gene cluster, TNFAIP3 and LCE3B/3C are also being associated with psoriatic gene susceptibility (Capon *et al.*, 2008; Chang *et al.*, 2008; de Cid *et al.*, 2009; Huffmeier *et al.*, 2006; Li *et al.*, 2009; Nair *et al.*, 2009).

2.1.2 Immunopathologic features of psoriasis.

2.1.2.1 Dendritic cells

Dendritic cells (DCs) are the professional antigen presenting cells (APC) that communicate message between innate and adaptive immune system. Myeloid dermal dendritic cells (DC.DC11c+) are present in abundance in psoriatic lesions and activate T cells for cytokine secretion. Another subtype of dendritic cells called TIP has capacity to produce proinflammatory molecules like TNF-α and iNOS (Nestle *et al.*, 2009a; Nestle *et al.*, 1994). In addition, DC.CD11c+ can produce the cytokines like IL-23 and IL-20 with potential to activate T cells and keratinocytes (Lee *et al.*, 2004a; Wang *et al.*, 2006). Moreover, mature DCs in dermal aggregates and the expression of lymphoid chemokines such as CCL19, CCL21, CXCL12 and CCL18 may promote T-cell activation (Lowes *et al.*, 2007a).

2.1.2.2 Cytokines and chemokines

The cutaneous and systemic overexpression of various proinflammatory cytokines in psoriasis is responsible for hyperproliferation of keratinocytesa and recruitment of the inflammatory cells with in the psoriatic plaques (Pietrzak *et al.*, 2008) Moreover, the interplay between genetic or environmental factors with various cytokines, chemokines and growth factors make psoriasis more complex than many other skin diseases.

IL-1: IL-1 is a proinflammatory cytokine with the capability to activate neutrophils, eosinophils and basophils, stimulate the production of cytokines like IL-2 and IFN by T cells and TNF, IL-6 and IL-8 by macrophages (Dinarello, 2002; Pietrzak *et al.*, 2008). Furthermore, IL-1 induces vascular endothelial cell adhesion molecules on keratinocytes and stimulates expression of KGF and GM-CSF in fibroblasts. In turn, these fibroblast-derived factors stimulate the proliferation and differentiation of keratinocytes (Werner and Smola, 2001). However, the role of IL-1 in the psoriasis is still controversial despite the direct correlation of IL-1 to the severity of psoriasis lesions (Mee *et al.*, 2006).

IL-2: IL-2 is a potent growth factor for T cells and it is secreted from pre-activated T cells. IL-2 stimulates the activity and proliferation of NK cells, monocytes and macrophages and stimulates the production of IFN, TNF, IL-6, and GM-CSF. High doses of IL-2 may induce psoriasis in predisposed patients (Kemmett *et al.*, 1990; Lee *et al.*, 1988; Pietrzak *et al.*, 2008).

IL-4: IL-4 is a Th2 cytokine and may be involved in the immune modulation from Th1 to Th2 and in exerting immune suppressive properties with antipsoriatic activity (Ghoreschi *et al.*, 2001; Ghoreschi *et al.*, 2003a). In addition, recent reports suggest involvement of IL-4 in the differentiation of Th9 lineage (Veldhoen *et al.*, 2008). Psoriatic lesions lack IL-4 or have very low level of expression (Uyemura *et al.*, 1993).

IL-9: IL-9 is secreted by activated Th2 cells and thought to be involved in Th2 associated disease. However, the regulation of IL-9 is different from other Th2 related cytokines like IL-4, IL-5 and IL-13 (Gessner *et al.*, 1993; Hauber *et al.*, 2004; Dardalhon *et al.*, 2008; Schmitt *et al.*, 1994; Veldhoen *et al.*, 2008). A recent study shows that IL-9 induces differentiation of Th17 cells and mediates Th17-driven inflammatory disease (Nowak *et al.*, 2009). IL-9 predominantly produced by Th17 cells, while IL-9 secretion by Th17 cells is regulated by IL-23. Addition of IL-9 together with IL-6 and TGF-β or alone greatly enhances the production of IL-9 from Th-17 cells in vitro (Elyaman *et al.*, 2009). IL-17RB binds IL-25, and regulates type-2 immune response and IL-9 production (Angkasekwinai *et al.,* 2010). In addition, adaptive Treg cells derived from encephalogenic T cells produce IL-9. IL-9 can be colonized with Treg cells within the tolerant allograft and is functionally important for allograft survival (Liu *et al.*, 2006; Lu *et al.*, 2006). Furthermore, IL-9 enhances the suppressive functions of Foxp3+ CD4+ Tregs in vitro (Elyaman *et al.*, 2009). Therefore, IL-9 is produced by T cells that play a role in both inflammation and immunosuppression.

IL-6: IL-6 is a key cytokine of Th17 family, produced by keratinocytes, fibroblasts, endothelial cells, and T cells and is involved in the growth and differentiation of dermal and epidermal cells. IL-6 is a major mediator of the host cell response to injury and infection, enhances the activation, proliferation and chemotactic behaviour of T lymphocytes towards dermis or epidermis (Baker and Fry, 1992). Moreover, it also enhances the proliferation and activation of B cells and macrophages in dermal infiltrate. It has been shown that number of different cells produce IL-6 in both non-lesional and lesional psoriatic skin. Furthermore, several external stimuli of the skin can induce the expression of IL-6 receptor causing the hyperproliferation of the skin in psoriatric patients (Pietrzak *et al.*, 2008; Toruniowa *et al.*, 1995).

IL-10: IL-10 is a pleiotropic cytokine secreted from different cells with anti-inflammatory properties. It inhibits the differentiation of naïve T cells towards Th1 and Th17 linage and inhibits the production of related cytokines (Asadullah *et al.*, 1998; Gu *et al.*, 2008). IL-10 also has been associated with Treg function in autoimmune diseases (Gangi *et al.*, 2005). There is no significant difference of IL-10 secretion and its receptor expression in psoriatic and non-psoriatic skin. Moreover, some authors showed complete lack of IL-10 receptor (IL-10R) in the keratinocytes of psoriatic skin (Nickoloff *et al.*, 1994).

IL-12: IL-12 (IL-12p70) consists of p35 and p40 subunits. It is secreted from various cells including Langerhans cells (LCs), monocytes, macrophages, and T cells (Yawalkar *et al.*, 1998). IL-12p70 is present in higher amount in psoriatic lesions expressed on dendritic cells, macrophages, neutrophils and CD15+ cells. However, there is no increased expression of p35 in psoriatic skin (Pietrzak *et al.*, 2008).

IL-17: IL-17 is the main cytokine of the Th17 family (The IL-17 family includes six cytokines, and the IL-17 receptor family is comprised of IL-17 receptors A, B, C, D and E) and it is known to stimulate the production of various mediators like IL-6, IL-8, GM-CSF and PGE2 in epithelial, endothelial, and fibroblastic cells. Moreover, IL-17 activates macrophages to produce TNF, IL-1, IL-6, IL-10, and IL-12. Both psoriatic and non-psoriatic skin express higher amount of IL-17 compared to normal skin. IL-17 mRNA is expressed on CD4+ and CD8+ T-cell clones derived from lesional psoriatic skin suggesting the functional role of IL-17 in psoriasis (Pietrzak *et al.*, 2008; Teunissen *et al.*, 1998b; Wilson *et al.*, 2007b).

IL-23: IL-23 consists of two subunits, p19 and p40, and plays a key role in peripheral inflammation. IL-23 activates Th17 cell to produce effector cytokines like IL-17 (Di Cesare *et al.*, 2009b). Both subunits of IL-23 are significantly up-regulated in psoriatic lesions compared to normal skin. IL-23 is secreted by keratinocytes, DCs, monocytes and T cells in psoriasis. It also has been shown that IL-23 is more important than IL-12 in the development of psoriasis (Di Cesare *et al.*, 2009b; Pietrzak *et al.*, 2008). In addition, the development of IL-23 immune response is independent of the IFN-gamma pathway (Langrish *et al.*, 2005).

IFN-γ: IFN-γ regulates the growth and differentiation of T cells, B cells, macrophages, NK cells, endothelial cells and fibroblasts. It upregulates proinflammatory mediators like IL-1, IL-6, IL-8, IL-12, IL-15, TNF, IP-10 (Pietrzak *et al.*, 2008). IFN- γ induces the expression of the adhesion molecules ICAM-1 on keratinocytes and endothelial cells, influencing the trafficking of T lymphocytes into lesional epidermis (Cabrijan *et al.*, 2009). However, the role of IFN-γ in psoriasis is still controversial.

TNF-α: TNF-α is a strong inflammatory cytokine and enhances the synthesis of IL-1, IL-6, GM-CSF, LIF, TGF, leukotrienes, PGE2 and ICAM, VCAM. It exerts its influences on the proliferation, activation and differentiation of many cell types (Ware, 2003). Its production is increased in the cutaneous inflammation as well as in psoriasis. Moreover, TNF-α upregulates CD40 and MHC-2 proteins on keratinocytes. TNF-α induces endothelial cells to express adhesion molecules and increase the production of VEGF leading to angiogenesis and erythema (Pietrzak *et al.*, 2008).

Chemokines: The key function of chemokines in psoriasis is to attract and recruit the leukocytes to the site of inflammation. Several chemokines such as TARC, MIG and IP-10 are known to be upregulated in psoriatic lesions and play an important role as chemoattractants for T cells at the inflamed dermal site. In addition, GMCSF, G-csf, RANTEES, KC, MIP, CCR6, CCR5, CCR4, CCR2, CXCR3, CXCR2, and CCL20 are found to be upregulated in psoriatic lesions. MIG and IP-10 are also synthesized by epidermal keratinocytes in response to IFN-γ. MCP-1 is a monocyte chemoattractant stimulated by IL-1, TNF-α, IFN-γ and TGF-α (Lowes *et al.*, 2007a; Nestle *et al.*, 2009b).

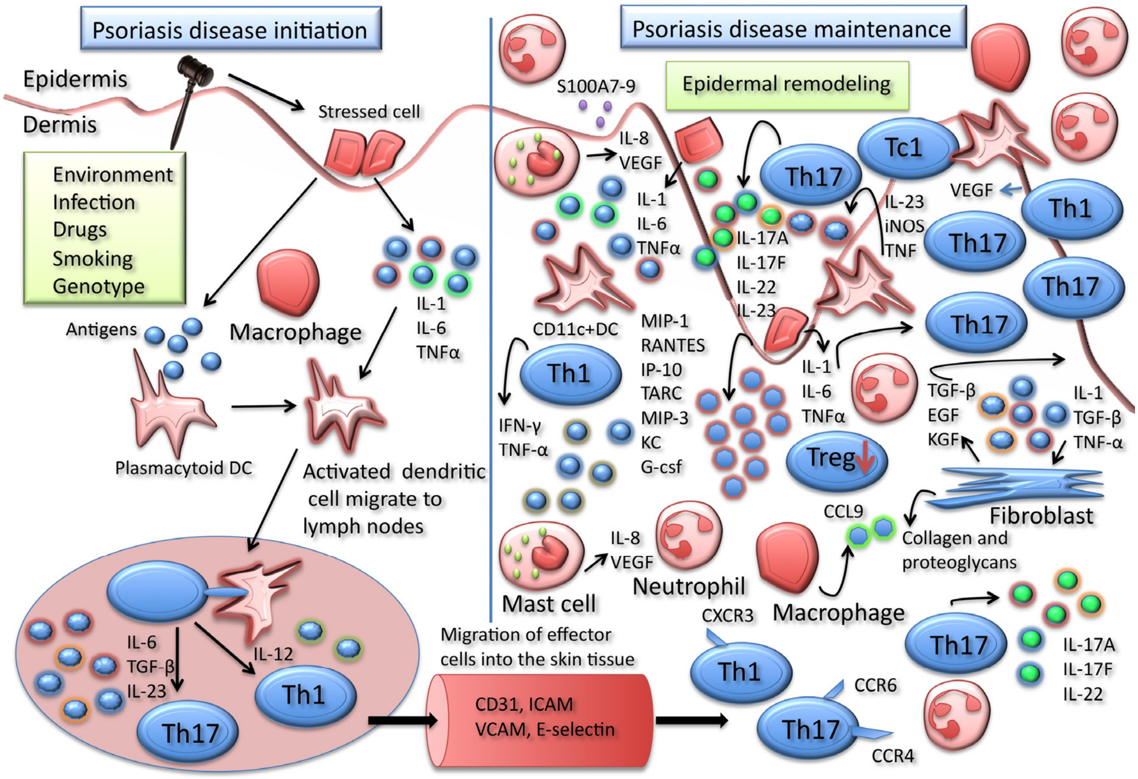

Figure 1: Main cytokine/chemokine events in the psoriasis

The development of psoriatic disease involves complex interaction of various cytokines and chemokines. Environmental (i.e. antigens) and genetic factors elicit the skin to produce cytokines (IL-1, IL-6, TNF-α) from stressed cells. These cytokines activate the plasmacytoid DC. After activation DCs move to the lymph node and induces the differentiation of different T cells including Th17 and Th1 in the presence of particular cytokines (IL-6, TGF-β, IL-23 or IL-12). Furthermore, these effector T cells move in to the skin through the capillaries, governed by integrin or selectin ligands (CD31, ICAM, VCAM and E-selectin). Different chemokine receptors (CXCR3, CCR6, and CCR4) attract T cells into the skin towards chemokine gradients. Secretion of various cytokines including IL-17A, IL-17F, IL-22, IL-23; IFN-γ and TNF-α produced by activated T cells

(Th17 or Th1) or DCs trigger the epidermal remodelling (including hyperkeratosis, parakeratosis and epidermal hyperplasia) as well as production of antimicrobial peptides and S100 proteins (S100A7-9) from keratinocytes. Other cell types including mast cell, fibroblast and macrophage are involved in the tissue organisation, endothelial activation and cluster formation by the down cascade of IL-8, VEGF, CCL9 and EGF. Accumulation of neutrophils in the epidermis or dermis occurs by chemokine attraction (CXCL8, CXCL1). Production of VEGF and TNF-α from keratinocytes stimulates angiogenesis. Activated keratinocytes secret miscellaneous chemokines (MIP-1, RANTES, IP-10, TARC, MIP-3, KC and G-csf) that contribute in tissue inflammation. In addition, secretion of IL-6 and TNF-α from keratinocytes inhibits the proliferation of Tregs and stimulates the development Th17 cells.

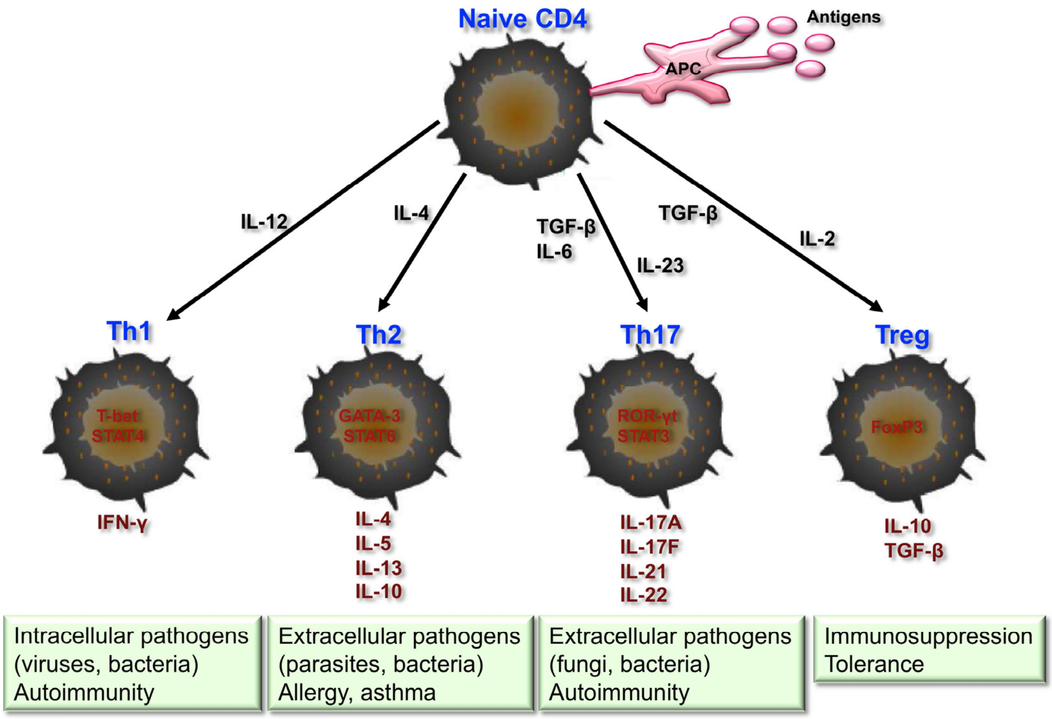

Figure 2: Differentiation of naïve CD4 T cells

Activated APC by antigens gives signal to the naïve CD4 T cell to differentiate in to the different cell types including Th1, Th2, Th17 and Treg cells. The differentiation of these cell types requires their unique cytokine and exert different down cascades.

2.1.2.3 The IL-23/Th17 axis

T cells in psoriatic lesions are polarized as T helper 1 (Th1) and T cytotoxic subsets (Tc1). Th1 polarized cytokines involve production of cytokines like IFN-γ and TNF-α through activation of STAT1 (McKenzie *et al.*, 2006). Th17 cells represent effector CD4+ T cells that produce special proinflammatory mediators, including IL-17A, IL-17F, IL-6, CCL20 and GM-CSF (Bettelli *et al.*, 2007a). IL-23 is an another unique cytokine that is composed of a p19 and p40 subunit expressed mainly on activated mouse and human monocytes, macrophages, dendritic cells, T cells, B cells, and endothelial cells. It is associated with the expansion of Th17 pathway for the production of IL-17A and other related proinflammatory cytokines (Di Cesare *et al.*, 2009b). As a Th17 family cytokine IL-23 leads to the development of psoriasis with characteristics of epidermal hyperplasia, acanthosis, hyperparakeratosis and erythema after intradermal injection into the mice, suggesting a prominent role in the pathogenesis of psoriasis (Di Cesare *et al.*, 2009b). IL-23 is involved in the maintenance while transforming growth factor (TGF)-β1 and IL-6 in the development of Th17 cells from naïve CD4+ T cells (McGeachy and Cua, 2007). Furthermore, RORγt and STAT3 are the key transcription factors which are involved in the development of Th17 differentiation. The production of IL-17A is mainly associated with Th17; however CD8+ T cells, γδ-TCR, and natural killer T cells can also produce IL-17A. The presence of IL-17 can be classified as IL-17A, IL-17F homodimers or IL-17A-IL-17F heterodimers and is thought to be involved in the expression of diverse proinflammatory mediators, including IL-6, IL-1β, GM-CSF, G-CSF, CXCL8, CXCL1 and CXCL10 from monocytes and epithelial cells (Lowes *et al.*, 2007a; Pietrzak *et al.*, 2008). The role of IL-23/Th17 axis in the pathogenesis of psoriasis further is supported by clinical data, in which therapeutically effective anti-TNF-α agents are able to down-regulate IL-23p19 and IL-12p40 mRNA levels as well as the inflammatory infiltrate in the psoriatic skin. Moreover, UV-therapy and cyclosporin A are also able to modulate the IL-23/Th17 axis. In addition, targeting the common subunit p40, IL-12 and IL-23 also show prominent role in autoimmune diseases including psoriasis. Recently, ustekinumab (CNTO-1275) has been approved as an anti-IL-12p40 mAb for the treatment for psoriasis and an anti-IL-17 mAb is in the pipeline for clinical trials (Nestle *et al.*, 2009a; Schon and Boehncke, 2005a). Thus, modulation or inhibition of the IL-23/Th17 axis offers a new concept to interfere with pathogenesis of psoriasis (Di Cesare *et al.*, 2009b; Lowes *et al.*, 2007a; McKenzie *et al.*, 2006; Pietrzak *et al.*, 2008). However, further research should provide a clear picture of IL-23/Th17 axis in the psoriasis disease pathogenesis and its involvement in other pathway of immune regulation.

2.1.2.4 Regulatory T cell

2.1.2.4.1 Phenotypic markers

Regulatory T cells (Tregs) are produced in the thymus and are observed in peripheral blood and in secondary lymphoid organs. Phenotypically natural Tregs express FoxP3, IL-2 receptor alpha (CD25), cell survival factor GITR and the T cell activation marker CTLA4, which is crucial for the maintenance of the suppressive function of these cells (Sakaguchi, 2005). Several other markers have also been identified on Tregs including CCR7, CD11a, CD39, CD45RA, CD45RO, ICAM-1, CD62L, CD103, LAG-3, Granzyme B, IL-10, MHC-II and perforin (Fehervari and Sakaguchi, 2004; Sakaguchi *et al.*, 2008)

Foxp3: The transcription factor Foxp3 is supposed to be vital in the development and function of Tregs. Foxp3 programs the development and function of CD4+CD25+ regulatory T cell and maintains immunological tolerance for autoimmunity, and transplantation (Tang and Bluestone, 2008; Wing and Sakaguchi, 2010). Expression of Foxp3 arises during the thymic development through high-affinity recognition of self-antigens (Sakaguchi *et al.*, 2008).

CD28: CD28 is a membrane glycoprotein constitutively expressed on majority of CD4+ T cells mature CD3+ thymocytes and plasma cells and required for T cell optimal stimulation and survival (June *et al.*, 1990; Lee *et al.*, 1990).

CD25: CD25 is a transmembrane protein which represents the low affinity alpha chain of the IL2 receptor. Majority of T cells, B cells, NK cells and monocytes expres CD25 on its surface (Sakaguchi *et al.*, 2008).

CTLA4: CTLA4 is the unique marker expressed on activated Tregs and regulates its function. Like CD28, CTLA4 binds to B7-1 and B7-2 but at rates reported to be 500- to 2,500-fold higher than those of CD28; thus giving CTLA4 a competitive advantage over CD28 (Paterson and Sharpe, 2010). It has been reported that anti-CTLA4 treatment inhibits the function of Tregs.

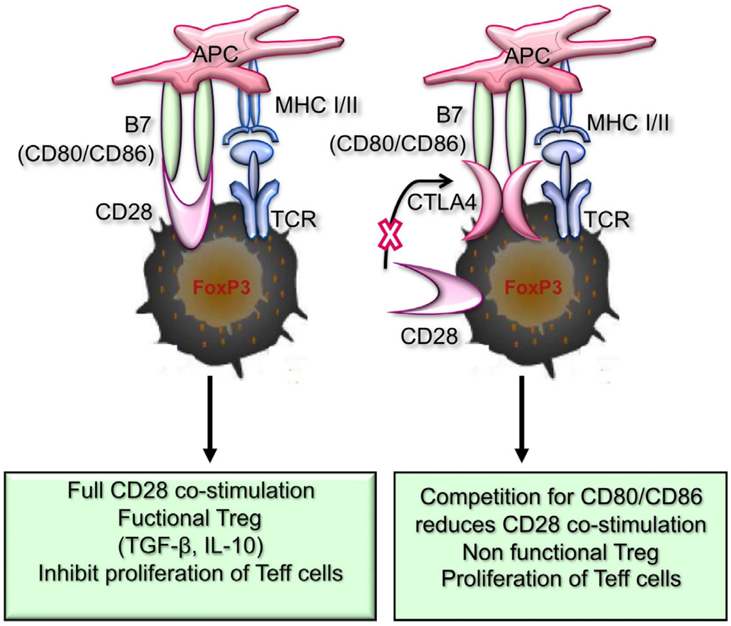

Figure 3: CTLA4 ligation arrests T cell function

CTLA4 ligation to the B7-CD80/CD86 inhibits Treg function by reducing CD28 co-stimulation

CD4: CD4 is a cell surface protein, expressed on T cells, thymocytes, macrophages, and dendritic cels. It forms synapse with MHC class II and APC for the recognition of foreign antigens (You *et al.*, 2004).

CD62L: CD62L belongs to selectin family (L-Selectin) as an adhesion protein and involve in leukocyte traffiking to secondary lymphoid organs. The expression of CD62L also reported on regulatory T cells for its traffiking (Ermann *et al.*, 2005; Fu *et al.*, 2004).

2.1.2.4.2 Function and generation

The main function of the regulatory T cell is to suppress the proliferation or activation of immune response and prevent pathological self-destruction i.e autoimmune disease (Wing and Sakaguchi, 2010). Foxp3+ Treg cells have the capacity to suppress various cell types including T cells, B cells, NK cells and DCs via different ways (Tang and Bluestone, 2008). The suppressive function of natural Treg cells that arise from thymus require cell-to-cell contact in vitro, whereas secretion of IL-10 and TGF-beta in vivo. However, suppressive functions of adaptive or induced Treg (iTreg) cells that arise extrathymically depend mainly on the secretion of the IL-10 and TGF-β. The suppressive capacity of Tregs also depends upon cytokine milieu, strength of stimulus and activation status of the APCs. Furthermore, the Treg response also depends upon site and other inflammatory environment (Bettelli *et al.*, 2006; Fantini *et al.*, 2004; Gangi *et al.*, 2005).

The role of TGF-β is not only limited for suppressive function of Tregs but recent observations revealed that TGF-β also has a critical role in the generation of Foxp3+ regulatory T cells**,** while the presence of IL-6 inhibits the conversion of T cell into Foxp3+ regulatory T cell and promotes Th17 cell development in vivo (Bettelli *et al.*, 2007a), which is programmed by orphan nuclear receptor RORγt (Manel *et al.*, 2008) rather than FoxP3**.** IL-2 is another cytokine that plays an important role in Treg generation. In psoriasis, peripheral blood Tregs are dysfunctional in suppressing the proliferative capacity of T responder (Tresp) cells (Sugiyama *et al.*, 2005a) and other T cell type that may reduce the capacity to contain pathogenic T cells, results in hyperproliferation of the psoriatic plaque. Taking together these findings may offer new therapeutic strategies by manipulating Tregs *in vivo*.

2.1.3 Platelet activating factor

Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a biologcially potent phospholipid mediator, playing a role in a variety of cellular signal transduction pathways. PAF is produced by and/or activates a wide variety of cells, including keratinocytes, platelets, mast cells, eosinophilic and polymorphonuclear leukocytes, monocytes, macrophages, endothelial cells, and fibroblasts (Ishii and Shimizu, 2000; Mallet and Cunningham, 1985; Walterscheid *et al.*, 2002). PAF is able to induce inflammatory events in the skin, including vascular changes and cellular infiltration. It also promotes the migration of leukocytes to sites of inflammation (Melnikova and Bar-Eli, 2007). Binding of the specific PAF receptor, a unique seven transmembrane spanning G-coupled protein induces a plethora of downstream effects, including activation of the mitogen-activated protein kinase pathway, activation of phospholipase, and the biosynthesis of COX2 and eicosanoids (including prostaglandins) and a variety of cytokines, including TNF-alpha, IL-1, IL-6, and IL-8 (Melnikova and Bar-Eli, 2007; Pei *et al.*, 1998). The activation of the epidermal PAF receptor results in increased biosynthesis of PAF (by an autocrine loop) (Maggi *et al.*, 1994; Peri *et al.*, 2003).

Several studies have implicated PAF in the pathogenesis of psoriasis. For instance, PAF may particularly contribute to the acute phase of leucotactic inflammation in patients with pustular psoriasis (Izaki *et al.*, 1996). Functionally, PAF enhances chemotaxis, aggregation and degranulation of peripheral blood polymorphonuclear leucocytes in vitro and increases vascular permeability in vivo (Braverman and Sibley, 1982). PAF may have priming effects for leucocytes and endothelial cells and enhance inflammatory events. Indeed, plasma levels of PAF were found being increased in patients with psoriasis, and a significant decrease in those levels was observed with clinical improvement after treatment (Andersen *et al.*, 1994). Moreover, elevated levels of arachidonic acid and eicosanoids and an elevated expression of human nonpancreatic phospholipase A2 was measured in psoriatic tissue (Andersen *et al.*, 1994). Phospholipase A 2 is one of the key enzymes for PAF formation since it enzymatically cleaves arachidonic acid from the sn-2 position of cell membrane phosphatidylcholine, and an acetyl residue is subsequently transferred to the free hydroxyl from acetyl-CoA to form biologically active PAF (Walterscheid *et al.*, 2002). Besides, PAF was identified and isolated in scales from psoriatic patients (Mallet and Cunningham, 1985; Ramesha *et al.*, 1987). Last but not least, Travers et al (Travers *et al.*, 1996; Travers *et al.*, 1995) suggested that PAF and its receptor play a direct role in keratinocyte pathophysiology.

The PAF pathway may influence T cell responses and favor the development of inflammatory Th17 pathway (Edwards and Constantinescu, 2009), in which T cells produce the proinflammatory cytokine IL-17A (IL-17) and IL-17F and, to a lesser extent, tumor necrosis factor (TNF) and IL-6 (Bettelli *et al.*, 2007b). Indeed, the expression of IL-17 appears to be increased in human autoimmune diseases including multiple sclerosis (Brucklacher-Waldert *et al.*, 2009; Fletcher *et al.*, 2009), rheumatoid arthritis (Heo *et al.*, 2009; Pernis, 2009), lupus erythematosus (Pernis, 2009), and psoriasis (Di Cesare *et al.*, 2009b) as well as in animal models of autoimmunity (Korn *et al.*, 2007).

2.2. Homing Receptors

Several receptors are expressed on lymphocytes for their recruitment or homing towards peripheral lymphoid organs like lymph nodes. Cutaneous lymphocyte antigen (CLA) is uniquely expressed in the skin. It is a product of fucosyl transferases VII dependent post-translational modification of Platelet (P)-selectin glycoprotein ligand 1 (PSGL-1) and interacts with endothelial (E)-selecti (Knibbs *et al.*, 1996). The expression of CLA depends on the local environment that likely includes cytokines like IL-4, IL-12, TGF-β and IL-10 (Lim *et al.*, 1999; Picker *et al.*, 1993; Sigmundsdottir *et al.*, 2004; Wagers and Kansas, 2000). In human up to 20% of peripheral blood T cells are CLA positive (Bos *et al.*, 1993). CLA positive T cells infiltrate to the skin, whereas, T cells infiltrating other (non-lymphoid) organs are mostly CLA negative (Picker *et al.*, 1991; Picker *et al.*, 1993).

Indeed, the major function of CLA is to control the influx and/or homing of memory T cells to the skin (Picker *et al.*, 1991). Moreover, the homing of T cells is also controlled by MHC-II, CD62 and CCR7 and the latter two may be co-expressed with CLA in significant number on peripheral T cells, including regulatory T cells (Tregs) (Campbell *et al.*, 2001). In addition, it also has been shown that homing of CD4+CD25+ regulatory T cell towards lymph node is controlled by the lymph node homing receptor L (leucocyte)-selectin (CD62L) and CCR7. Moreover, Tregs induced by UV radiation have been shown to express CD62L as well, but like other T cells not E-and P (platelet)-selectin(Schwarz *et al.*, 2004). Their homing is also controlled by expression of CCR7 and MHC-II on their surface.

2.3. Psoralen plus UVA photochemotherapy

Psoralen plus UVA **(**PUVA) is used since the 70ies in the Western world as a highly effective standard dermatological treatment for various skin conditions, including psoriasis. However, 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 focused on the binding of psoralen to DNA, in which pyrimidine bases are the main targets for a photochemical reaction (Caffieri, 2002). 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. However, similar to UV radiation (Duthie *et al.*, 1999), PUVA has also been known for long time to have profound immunosuppressive properties, potentially initiated through cytokine release after both DNA and/or cell membrane alterations (Wolf *et al.*, 2006). For instance, Vallat et al.(Vallat *et al.*, 1994) have shown that PUVA can exert distinct suppressive effects on infiltrating immune cells in psoriatic skin, with virtually total elimination of IL-2 receptor+ T cells in some patients. In comparison to 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.

2.4. K5.hTGF-β1 transgenic mice

In our study we used K5.hTGF-β1 transgenic mice expressing human wild type (latent) TGF-β1 in the basal layer of the epidermis as psoriasis model (Li *et al.*, 2004) **(Fig.1)**. K5.hTGF-β1 transgenic mice 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 cytotokine abnormalities that typically occur in human Th1-type inflammatory skin disorders, such as psoriasis (Schon and Boehncke, 2005b). The skin lesions in K5.hTGF-β1 transgenic mice are characterized by epidermal hyperproliferation, massive infiltration of neutrophils, CD4+ T 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 (Schon, 2008), rendering this model very attractive to study anti-psoriatic 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 (Han *et al.*, 2010).

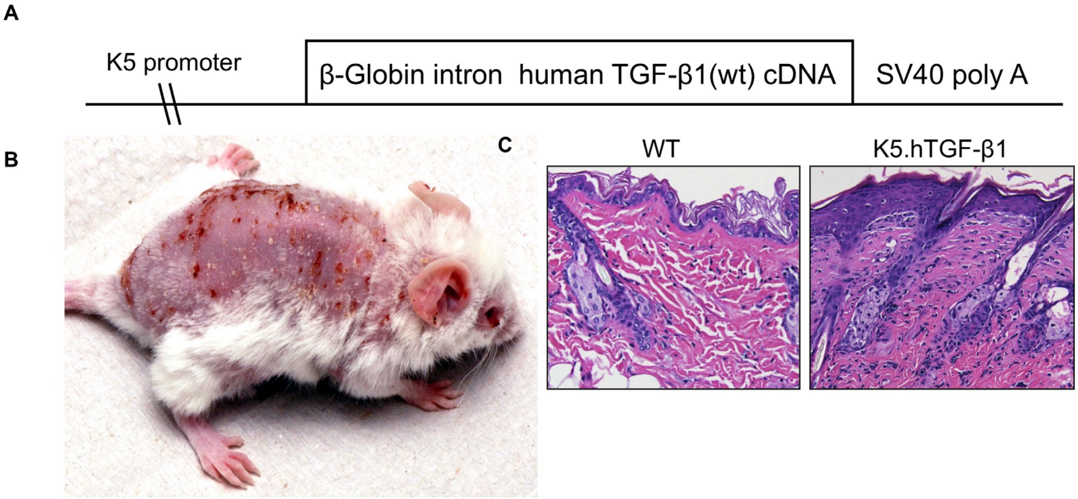

Figure 4: Generation of K5.hTGF-β1 transgenic mice and its phenotype

(A) The K5.hTGF-β1 transgene. (B) Macroscopic presentation of K5.hTGF-β1 mice. (C) Microscopic presentation of H&E-stained sections of dorsal skin of WT and K5.hTGF-β1 mice (Mice provided by Michael P. Schön[†,‡], Katrin Wallbrech[‡], Kai Michaelis[‡] and Xiao-Jing Wang[¶]; [†]Department of Dermatology, University Medical Center Göttingen, Göttingen, Germany. [‡]Rudolf Virchow Center for Experimental Biomedicine and Department of Dermatology, University of Würzburg, Würzburg, Germany [¶]Departments of Pathology, Otolaryngology, Dermatology, and Craniofacial Biology, Head and Neck Cancer Research, University of Colorado Denver, USA).

3. MAJOR AIMS OF THE STUDY

Study part 1. To study the IL-23/Th17 and Treg axis in 8-methoxypsoralen plus UVA (PUVA) treatment of psoriasis.

Despite the introduction of new therapeutic strategies such as tumor necrosis factor (TNF)-α inhibiting biologics or anti-IL-12/23 antibody treatment, 8-methoxypsoralen plus UVA (PUVA) photochemotherapy and other forms of phototherapies have kept their prominent places in the dermatological treatment armamentarium of psoriasis and many other skin diseases. However, the mechanisms by which PUVA leads to clearance of skin lesions are still not well understood. 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.

Study part 2. To study the role of platelet activating factor (PAF) and its receptor on Th17 pathway in the pathogenesis of psoriasis.

Previous studies have implicated platelet-activating factor (PAF), a highly active lipid mediator in the pathogenesis of psoriasis. We were therefore interested to investigate whether the PAF pathway may influence T cell responses and favour the development of the inflammatory Th17 pathway, in which T cells produce the proinflammatory cytokine IL-17A (IL-17), IL-17F and IL-12 and, to a lesser extent, tumor necrosis factor (TNF) and IL-6. We thus tested the hypothesis that PAF/PAF receptor interaction plays a role in the induction of the psoriatic phenotype and Th17 cytokine abnormalities in K5.hTGF-β1 transgenic mice, a model that reflects close resemblance with human psoriasis.

4. RESULTS OF STUDY PART 1

The results of study part 1 have been published in The Journal of Immunology in 2010:

Tej Pratap Singh, Michael P. Schön, Katrin Wallbrech, Kai Michaelis, Beate Rinner, Gerlinde Mayer, Ulrike Schmidbauer, Heimo Strohmaier, Xiao-Jing Wang, Peter Wolf

8-methoxypsoralen plus UVA therapy acts via inhibition of the IL-23/Th17 axis and induction of Foxp3+ Tregs involving CTLA4 signaling in a psoriasis-like skin disorder.

The Journal of Immunology 2010; 184(12):7257-7267

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 minimal phototoxic dose (MPD) as the starting dose for PUVA treatment (Legat *et al.*, 2004). 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 to 72 hrs after psoralen administration. In order 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 sub-phototoxic dose of 0.25 J/cm^2 or a near-phototoxic dose of 0.50 J/cm^2 twice a week over 4 weeks. These doses are in the range of MPD values used for starting PUVA therapy in patients. **Fig. 5A** shows that both PUVA doses initially caused skin swelling in the transgenic mice in a dose-dependent manner, similar than in wild type mice (**Fig. 6B-D**). However, during the 4-week treatment regimen this swelling resolved and PUVA then prevented a further increase of the thickened skin (**Fig. 6A**) that occurred in transgenic mice compared to wild type mice. Likewise, both PUVA doses significantly improved the severity of skin disease in the mice (**Fig. 5B**). The macroscopic improvement of the skin status (**Fig. 5C**) of the mice was paralleled by a decrease of histological alterations (**Fig. 5D, E**). 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. 5A, B, D and E**). Notably, PUVA treatment signficantly prolonged the survival of mice (**Fig. 5F**), a result that is consistent with observations in humans, in whom PUVA can be life saving for severe cases of erythrodermic or pustular psoriasis.

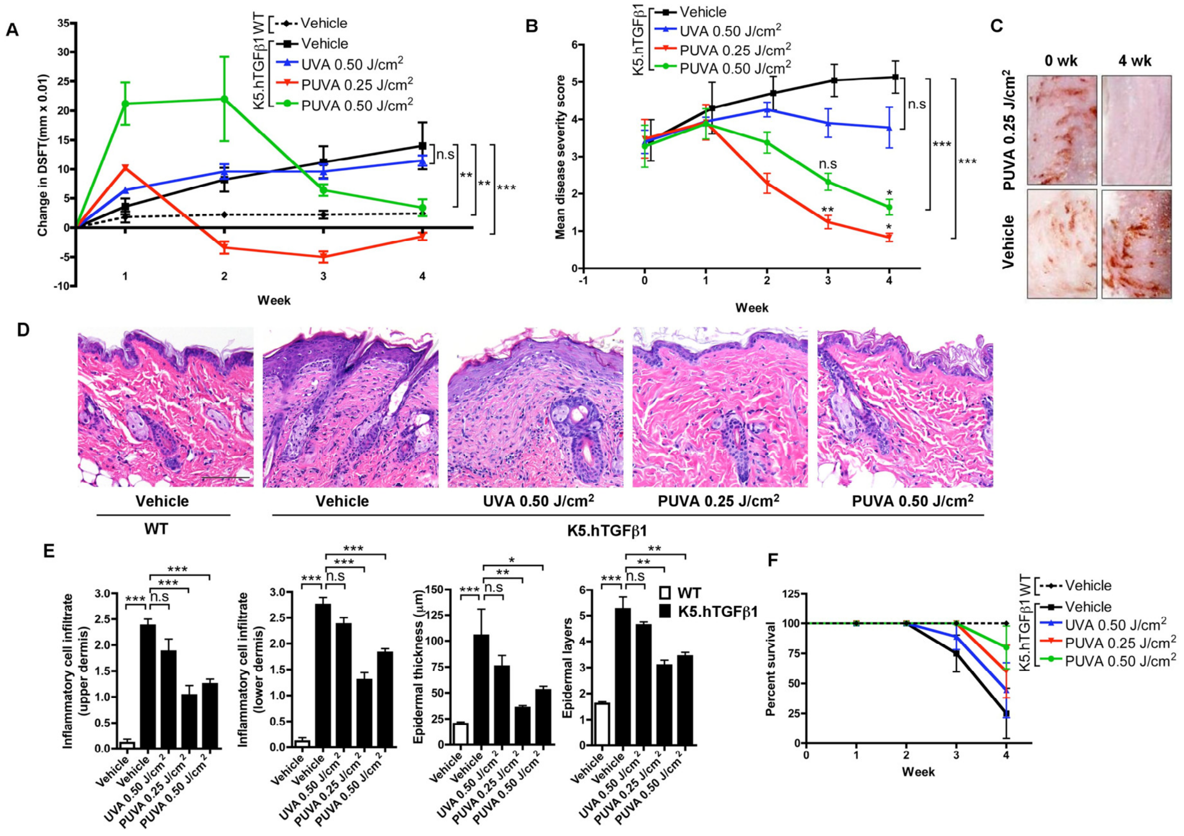

Figure 5: PUVA treatment clears psoriatic skin lesions in K5.hTGF-β1 transgenic mice.

K5.hTGF-β1 or wild type (WT) mice were topically treated on their shaved back skin twice a week for 4 weeks with either 8-methoxypsoralen 15 min before exposure to a sub- or near-phototoxic dose of UVA (PUVA 0.25 J/cm^2 or 0.50 J/cm^2), (mimicking a clinical PUVA regimen), UVA (0.50 J/cm^2) radiation alone or vehicle (ethanol) alone. ***(A)*** *Double skin fold thickness (DSFT) was measured before the first treatment and 48 hours after the second treatment of every week.* ***(B)*** *A specific score (composed of a rating from 0 to 3 for erythema, infiltration, and scaling) was used to monitor macroscopic disease severity in the mice. **, *, n.s, 3 wk or 4 wk versus 0 wk.* ***(C)*** *Presentation of a representative PUVA (0.25 J/cm^2) or vehicle-treated K5.hTGF-β1 transgenic mouse before and after 4 weeks of treatment (original magnification x 100).* ***(D)*** *Representative images of H&E-stained sections from dorsal skin of a mouse of the different treatment groups (scale bar 100 µm; original magnification x 100).* ***(E)*** *Evaluation of histological features, including inflammatory infiltrate (0, no; 1, mild; 2, moderate; 3, severe) in the upper or lower dermis, epidermal thickness and number of epidermal layers.* ***(F)*** *PUVA prolongs survival of the K5.hTGF-β1 mice ($p \leq 0.05$ comparing PUVA 0.50 J/cm^2 -treated versus vehicle-treated transgenic mice). Data shown are from one representative experiment, with n=5 per treatment group (A, B, F) or pooled from two experiments, with* *n=4-9 mice per treatment group (E). *, p≤0.05; **, p≤0.01; ***, p≤0.001. n.s, not significant.*

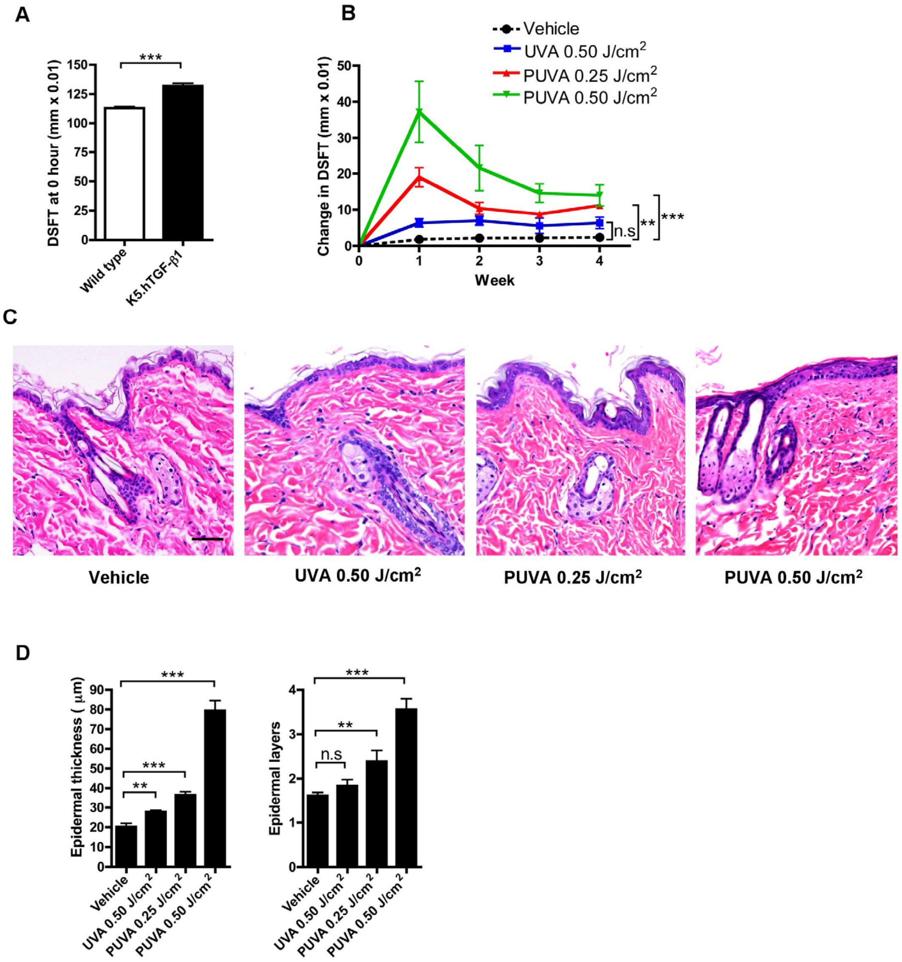

Figure 6: Effect of PUVA on skin thickness of wild type mice.

*Wild type (WT) mice were topically treated on their shaved back skin twice a week for 4 weeks with either 8-methoxypsoralen 15 min before exposure to a sub- or near-phototoxic dose of UVA (PUVA 0.25 J/cm^2 or 0.50 J/cm^2) or UVA (0.50 J/cm^2) radiation alone or vehicle (ethanol) alone. (A) Double skin fold thickness (DSFT) was measured before the first treatment and (B) 48 hours after the second treatment of every week. (C) Representative images of H&E-stained sections from dorsal skin of a mouse of the different treatment groups (scale bar 50 µm). (D) Evaluation of histological features, including epidermal thickness and number of epidermal layers. Data shown are from one representative experiment, with n=5 per treatment group. **, p≤0.01; ***, p≤0.001*

PUVA inhibits IL-23/Th17 axis in K5.hTGF-β1 transgenic mice

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 to wild type 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 RORgammat (ROR-γt) and STAT3 transcription factors in the skin of transgenic mice (**Fig. 7A**). These results were paralleled by the depression of serum protein levels of IL-17, IL-12p70 (i.e., the bioactive heterodimer comprised of the p40 and p35 IL12-subunits), IL-23, IL-6, IL-1a and IL-1b (**Fig. 7B**). UVA given alone had no significant effect on these factors. Immunohistochemical staining with anti-IL23p19 antibody 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. 7C and D**) as well as STAT3 in the dorsal skin (**Fig. 13**). 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-week PUVA treatment regimen. PUVA decreased IL-17A and IL-17F producing CD4+ cells in transgenic mice in a dose-dependent fashion by up to approximately 2-fold and 6-fold, respectively (**Fig. 7E**). We also found that PUVA downregulated abnormally elevated serum levels of G-CSF, GM-CSF, MCP-1, RANTES, KC, IP-10, MIP-1a, IL-9, TNF-α, and IFN-γ (**Fig. 8A**). In contrast, PUVA increased protein levels of IL-4, IL-10, and IL-13 in the serum. IL-2, IL-5, IL-7, an 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 mRNA expression in the skin, consistent with the decrease of those cytokines in the serum (**Fig. 8B**). In addition, PUVA decreased the IFN-γ content of CD4+ splenocytes in a dose-dependent fashion by up to more than 10-fold in transgenic mice (**Fig. 10**).

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. 8A**), but the levels after PUVA exposure were still significantly higher in transgenic mice compared to wild type mice (approximately 6-fold at the PUVA dose of 0.25 J/cm^2 and 3-fold at the PUVA dose of 0.50 J/cm^2). Immunohistochemical staining of the skin revealed that there was no marked difference in the intensity of TGF-β staining between PUVA-treated and non-treated transgenic mice in the epidermis (**Fig. 11**), 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- βexpression in the skin appeared to be specific since other genes such as keratin 5, keratin 14, filaggrin, and loricrin were not significantly affected by the treatment **(Fig. 12)**. Collectively, these results indicated that PUVA treatment downregulates the IL-23/Th17 pathway and Th1 cytokines but upregulates Th2 ctyokines 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. 9**), K5.hTGF-β1 transgenic mice were injected twice weekly over a 4-week period with an anti-IL-17 monoclonal antibody. The anti-IL-17 treatment stopped the disease progression in the K5.hTGF-β1 mice, compared to injection with an isotoype control antibody (**Fig. 9A-C**). However, anti-IL-17 antibody injection did not affect IL-10 and Foxp3 mRNA expression in the dorsal skin of the mice (**Fig. 9D**).

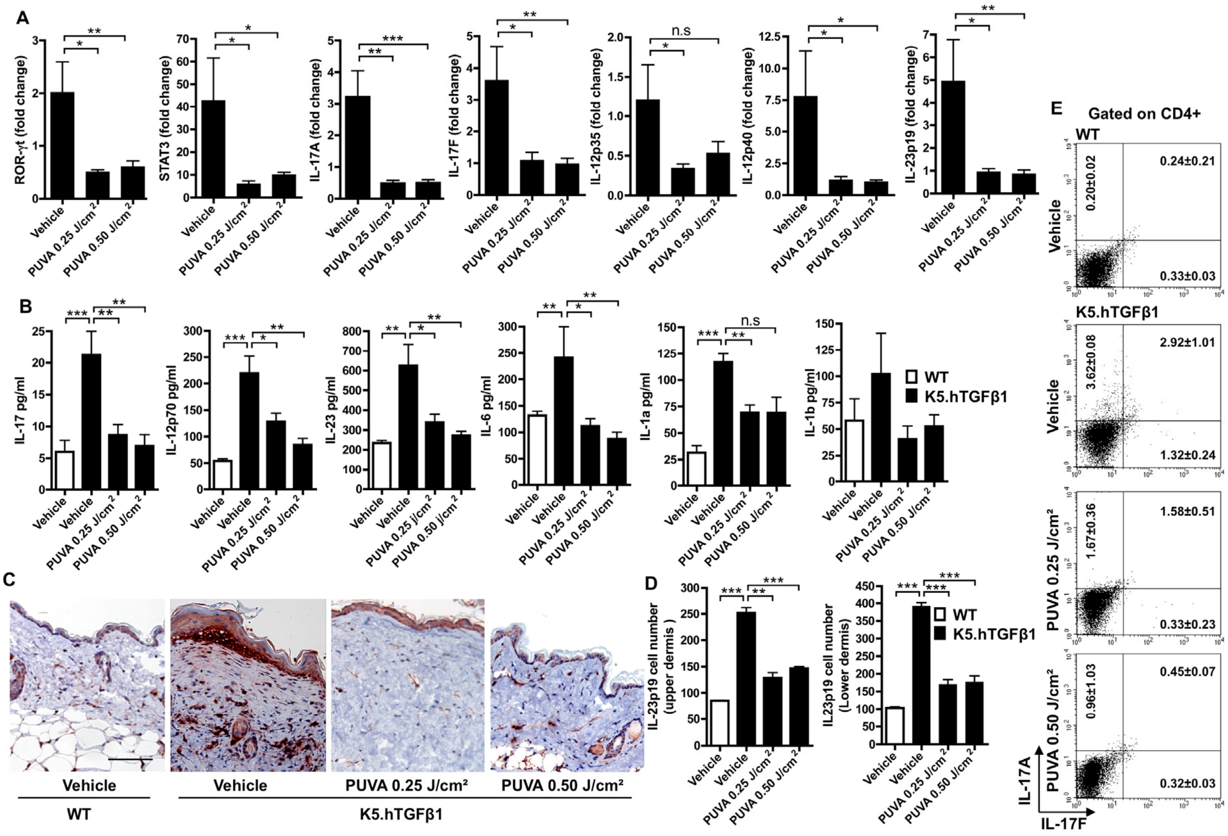

Figure 7: PUVA treatment downregulates transcription factors and cytokine profile of the IL-23/Th17 pathway

K5.hTGF-β1 transgenic or wild type (WT) mice were treated, as described in Figure 5. At the end of the 4-week treatment regimen tissue or serum samples were collected and analyzed. ***(A)*** *Quantitative RT-PCR was performed with RNA isolated from individual* *mouse dorsal skin for transcription factors ROR-γt and STAT3 and transcript encoding cytokines IL-17A, IL-17F, IL-12p35, IL-12p40, and IL-23p19.* ***(B)*** *IL-17, IL-12p70, IL-23, IL-6, IL-1a, and IL-1b serum levels were determined either by ELISA or bead immuno assay.* ***(C)*** *Photographs of immunohistochemical staining for IL-23p19 in dorsal skin from mice of the different treatment groups (scale bar 100 µm).* ***(D)*** *Quantitative analysis of IL-23p19 staining. IL-23p19-positive cells were microscopically counted in 10-15 consecutive visual fields in the upper or lower dermis (final magnification, x200).* ***(E)*** *Intracellular cytokine staining for IL-17A and IL17F were performed on pooled cultured spleen single cell suspension collected from K5.hTGF-β1 mice treated with vehicle or PUVA. Cells were stimulated and analyzed, as described in materials and methods. Results shown are gated on the CD4+ population. Data shown are from two pooled experiments, with a total of n=4-9 (A, B, D) or n=6 mice per treatment group (E). *, p≤0.05; **, p≤0.01; ***, p≤0.001. n.s, not significant.*

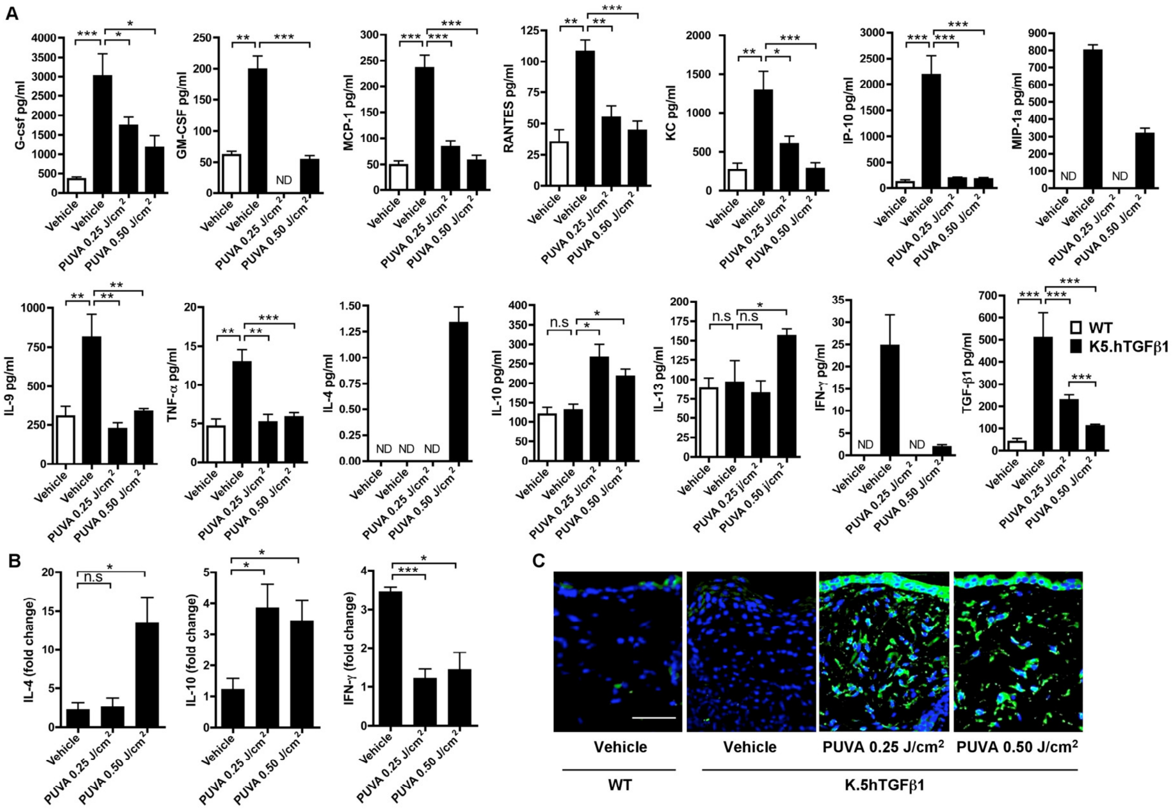

Figure 8: PUVA treatment influences chemokine and Th1/Th2 cytokine profile *K5.hTGF-β1 transgenic or wild type (WT) mice were treated, as described in Figure 5. At the end of the 4-week treatment regimen serum or tissue samples were collected and analyzed.* ***(A)*** *G-CSF, GM-CSF, MCP-1, RANTES, KC, IP-10, MIP-1a, IL-9, TNF-**α**, IL-4, IL-10, IL-13, IFN-γ, and TGF-β1 (mouse + human) serum levels were determined either by ELISA or bead immuno assay.* ***(B)*** *Quantitative RT-PCR was performed with*

RNA isolated from individual mouse dorsal skin for IL-4, IL-10, and IFN-γ mRNA expression. ***(C)*** *Immunofluorescent staining for IL-10 in dorsal skin from mice of the different treatment groups (green, IL-10 staining; blue, DAPI counterstaining; scale bar 100 μm). Data shown are from two pooled experiments, with n=4-9 mice per treatment group (A, B). *, p≤0.05; **, p≤0.01; ***, p≤0.001. ND., not detectable. n.s, not significant.*

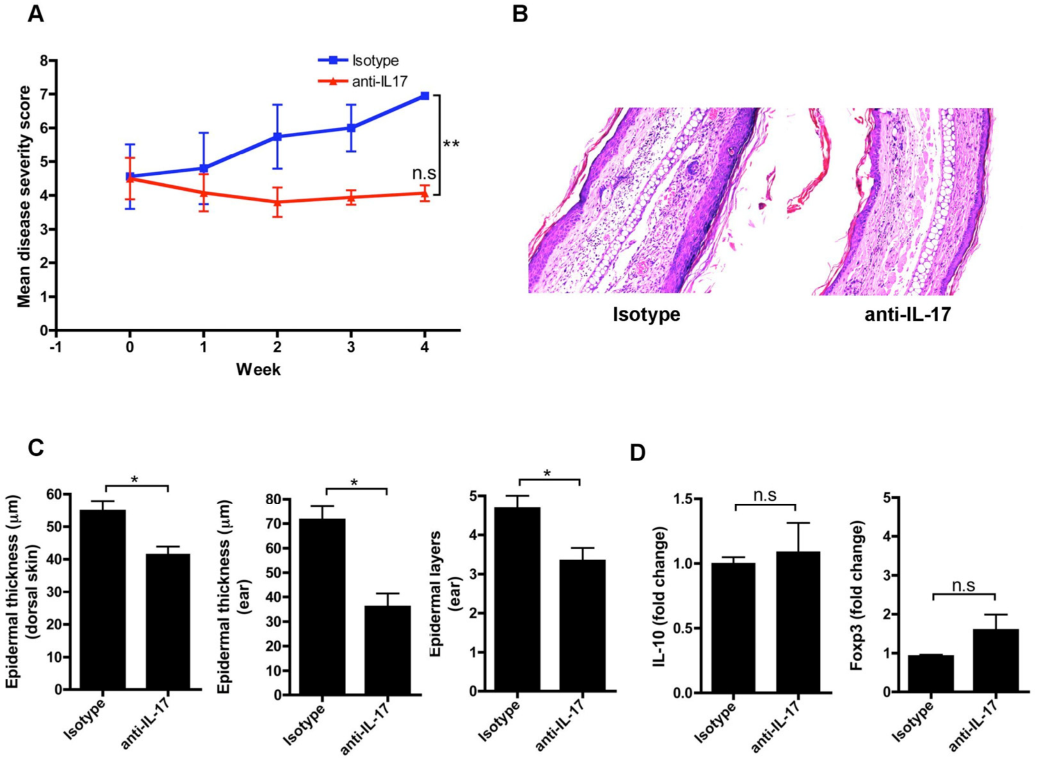

Figure 9: Blockade of IL-17 stops progression of psoriatic skin disease

K5.hTGF-β1 transgenic mice were injected i.p. twice a week with anti-IL-17 monoclonal antibody or an isotype control antibody. ***(A)*** *Disease severity in the mice.n.s, 4 wk versus 0 wk.* ***(B)*** *Representative images of H&E-stained sections of ears from the mice (scale bar 100 μm).* ***(C)*** *Epidermal thickness and epidermal cell layers, as measured in the H&E-stained sections of ears or dorsal skin.* ***(D)*** *IL-10 and Foxp3 mRNA expression in the dorsal skin of the mice. Data shown are from one representative experiment, with n=5 mice per treatment group. *, p≤0.05; **, p≤0.01. n.s, not significant.*

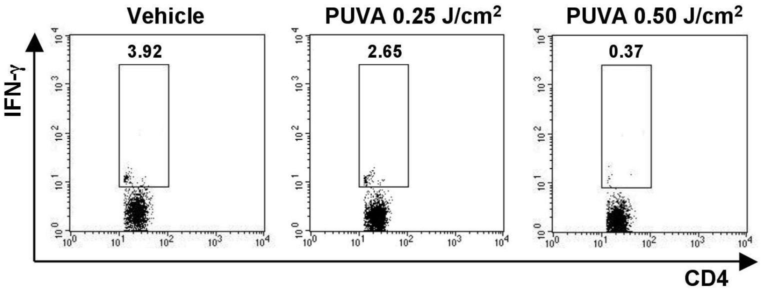

Figure 10: PUVA decreases IFN-γ secreting CD4+ T cell population

K5.hTGF-β1 transgenic mice or wild type (WT) mice were topically treated on their shaved back skin twice a week for 4 weeks with either 8-methoxypsoralen 15 min before exposure to a sub- or near-phototoxic dose of UVA (PUVA 0.25 J/cm^2 or 0.50 J/cm^2) or vehicle (ethanol) alone. Intracellular cytokine staining for IFN-γ was performed on pooled cultured spleen single cell suspension collected from K5.hTGF-β1 TG mice treated with vehicle or PUVA. Cells were stimulated and analyzed, as described in materials and methods. Results shown are gated on the CD4+ population. Data shown are from one representative experiment, with n=3 mice.

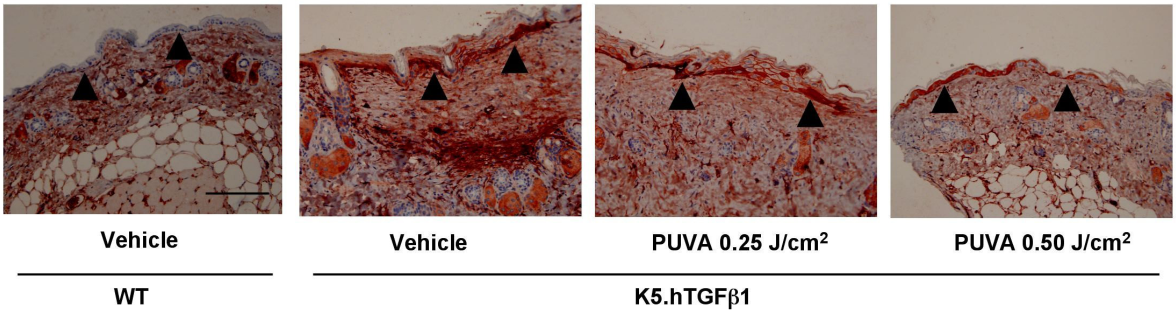

Figure 11: Expression of (h)TGF-β in the dorsal skin of mice

K5.hTGF-β1 transgenic mice or wild type (WT) mice were topically treated on their shaved back skin twice a week for 4 weeks with either 8-methoxypsoralen 15 min before exposure to a sub- or near-phototoxic dose of UVA (PUVA 0.25 J/cm^2 or 0.50 J/cm^2) or vehicle (ethanol) alone. Immunohistochemical staining for TGF-β in the dorsal skin from mice of the different treatment groups at week 4 (scale bar 100 μm). Arrows depict the epidermal-dermal junction. Note that (h)TGF-β is strongly expressed in the epidermis of transgenic mice (but not WT mice), independent of treatment.

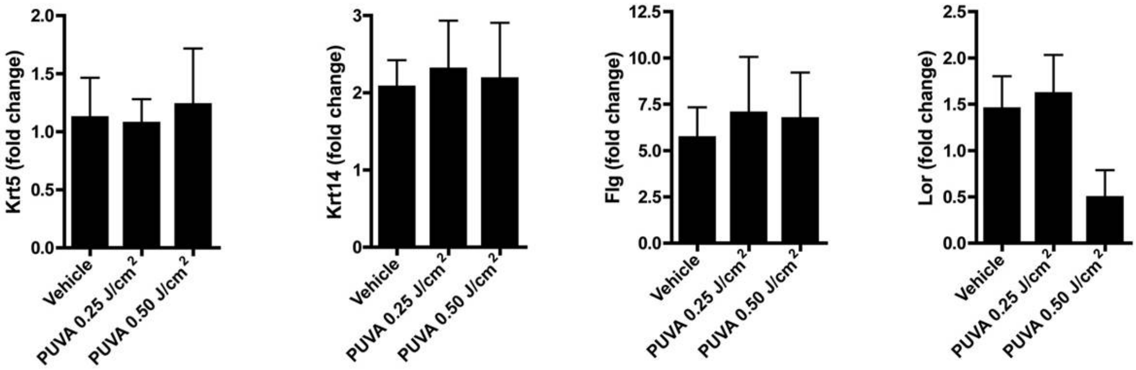

Figure 12: PUVA does not influence the expression of Krt5, Krt14, Flg and Lor

K5.hTGF-β1 transgenic mice or wild type (WT) mice were topically treated on their shaved back skin twice a week for 4 weeks with either 8-methoxypsoralen 15 min before exposure to a sub- or near-phototoxic dose of UVA (PUVA 0.25 J/cm^2 or 0.50 J/cm^2) or vehicle (ethanol) alone. Real time PCR analysis was performed on the dorsal skin for keratin 5 (Krt5), keratin 14 (Krt14), filaggrin (Flg) and loricrin (Lor) as described in material and methods.

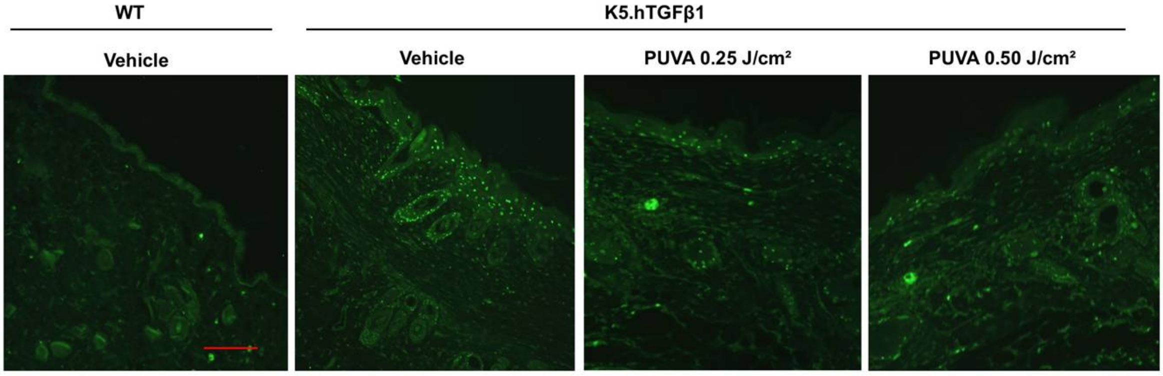

FIGURE 10. Expression of STAT3 in the dorsal skin of mice

K5.hTGF-β1 transgenic mice or wild type (WT) mice were topically treated on their shaved back skin twice a week for 4 weeks with either 8-methoxypsoralen 15 min before exposure to a sub- or near-phototoxic dose of UVA (PUVA 0.25 J/cm^2 or 0.50 J/cm^2) or vehicle (ethanol) alone. Immunofluorescent staining for STAT3 in the dorsal skin from mice of the different treatment groups at week 4 (scale bar 100 µm).

PUVA-inuced IL-10 producing Foxp3+ T regulatory cells 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) (Chen *et al.*, 2003; Fantini *et al.*, 2004; Liu *et al.*, 2003). To study the role of Tregs in the K5.hTGF-β1 mice, we first compared CD4+CD25+Foxp3+ Treg populations from lymph node cells or splenocytes between wild type and K5.hTGF-β1 transgenic mice and did not find any significant differences **(Fig. 14A**). 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/cm^2 compared to 0.25 J/cm^2 (**Fig. 14A**). Quantitative RT-PCR analysis of RNA extracted from dorsal skin revealed a 3.5-fold increase of Foxp3 transcript in PUVA-treated compared to vehicle-treated K5.hTGF-β1 transgenic mice (**Fig. 14D**). In addition, PUVA treatment increased the percentage of CD4+CD25+Foxp3+ IL-10+ Tregs among splenocytes in transgenic mice, compared to vehicle treatment (**Fig. 14B**). 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. 14E**). Furthermore, PUVA did increase IL-10 positivity of keratinocytes in the epidermis and cells in the inflammatory infiltrate of the dermis (**Fig. 8C**), consistent with the result of a previous study (Wolf *et al.*, 2006). The exact type and origin of PUVA-induced II-10 positive cells in the dermis remains unclear at present, however, they may represent macrophages, dendritic cells and/or even certain T cells (Boonstra *et al.*, 2006; Maeda *et al.*, 2008). However, there was no significant difference neither in IL-10 expression (**Fig. 8B**) nor IL-10 protein levels in the skin (**Fig. 8C**) and serum (**Fig. 8A**) between vehicle-treated wild type and transgenic mice.

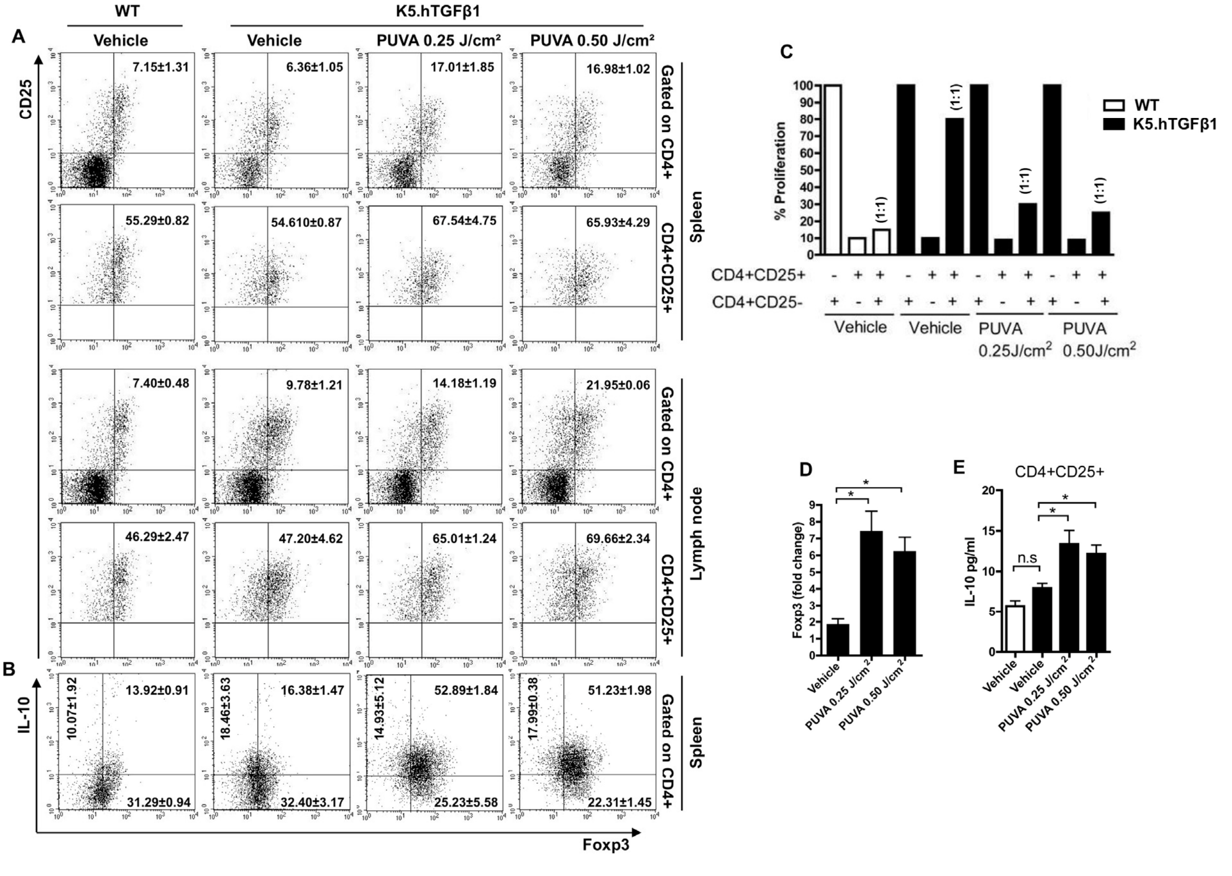

Figure 14: Phenotypic and functional analysis of Tregs

K5.hTGF-β1 transgenic or wild type (WT) mice were treated, as described in Figure 5. At the end of the 4-week treatment regimen skin, spleen, and lymph nodes samples were collected. ***(A)*** *FACS of pooled single cell suspension from spleen or lymph nodes of WT or K5.hTGF-β1 mice, stained with monoclonal antibodies to CD4, CD25, and Foxp3.* ***(B)*** *FACS analysis of CD4, Foxp3 and IL-10 in isolated and proliferated CD4+CD25+ Treg cells.* ***(C)*** *Proliferation assay of pooled CD4+CD25+, CD4+CD25- or their co-culture from vehicle-treated WT or K5.hTGF-β1 mice, or PUVA-treated K5.hTGF-β1 mice. Each cell subset was cultured either in duplicate alone (1 x 10^5) or with an equal number (1:1) of CD4+CD25+ and CD4+CD25- cells. Cells were stimulated and analyzed, as described in materials and methods. CD4+CD25- proliferation considered as 100%.* ***(D)*** *Quantitative RT-PCR analysis for Foxp3 mRNA expression in dorsal skin.* ***(E)*** *Supernatant of proliferated CD4+CD25+ T cells was collected at 72 h and an IL-10 specific ELISA was performed. Data shown are from two pooled experiments, with n =6 total mice (A, B) or n=4-9 per treatment group (D), or one representative experiment, with n=3 mice (C, E) per treatment group. *, p≤0.05.*

It has been shown previously that Tregs from patients with psoriasis were dysfunctional in suppressing the proliferation of T responder cells (Sugiyama *et al.*, 2005a). 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 wild type nor PUVA-treated or untreated K5.hTGF-β1 transgenic mice proliferated when stimulated with plate-bound anti-CD3 antibody alone or combined with plate-bound anti-CD3 and soluble anti-CD28 antibody. However, T responder cells had full capacity of proliferation upon stimulation with plate-bound anti-CD3 and soluble anti-CD28 antibody. 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 sub-maximal disease development. To investigate whether PUVA is able to induce Tregs with suppressive activity, we performed co-culture experiments with Tregs and T responder cells at a 1:1 ratio (**Fig. 14C**). Tregs from vehicle-treated K5.hTGF-β1 transgenic mice failed to suppress the proliferation of T responder cells. In contrast Tregs from K5.hTGF-beta1 transgenic mice treated with PUVA were able to suppress the proliferation of T responder cells in a dose-dependent fashion to levels that are comparable to wild type cells (**Fig. 14C**). This is consistent with the findings by Schwarz et al.(Schwarz *et al.*, 2004), 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.

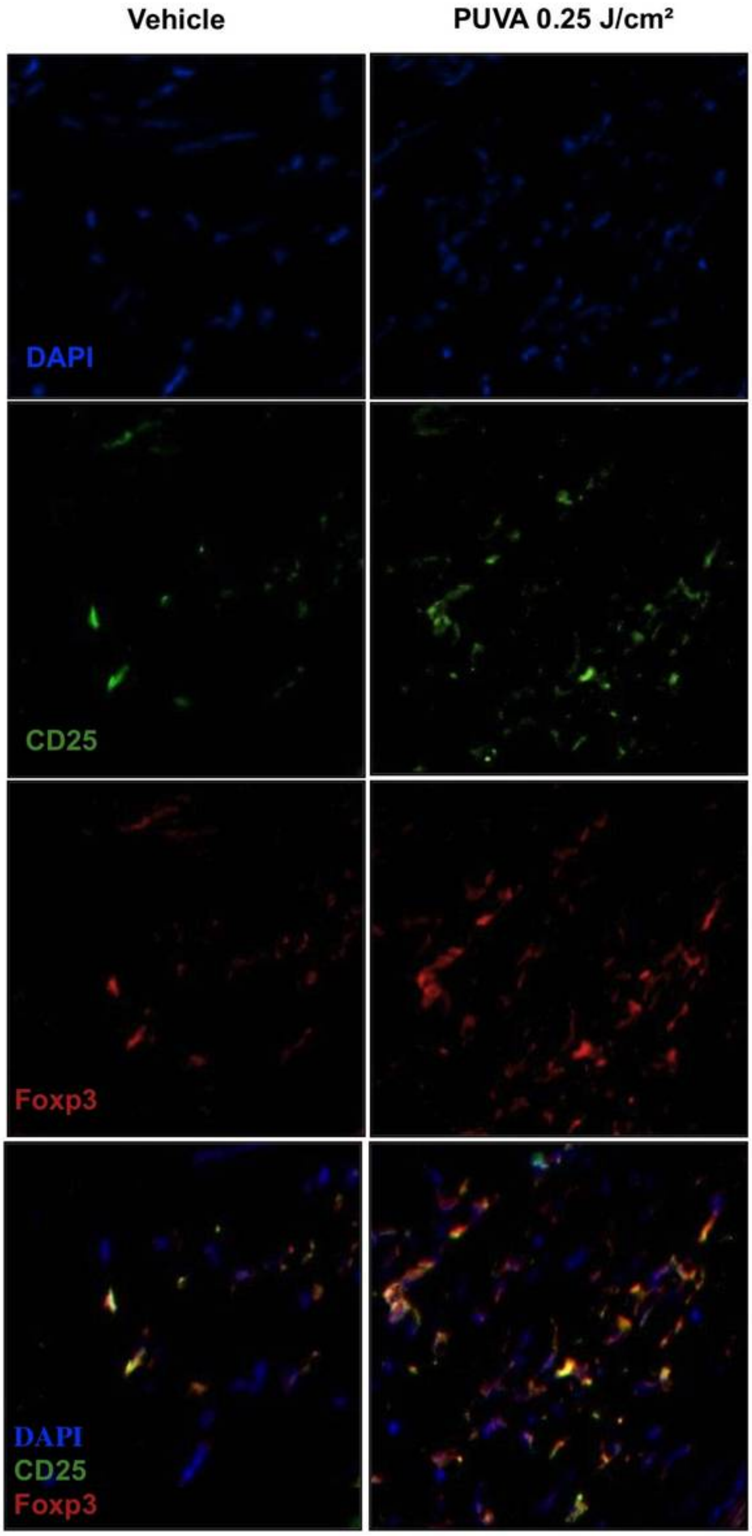

Figure 15: PUVA increases Foxp3+CD25+ T cell in the skin

K5.hTGF-β1 mice were topically treated on their shaved back skin twice a week for 4 weeks with either 8-methoxypsoralen 15 min before exposure to a sub-phototoxic dose of UVA (PUVA 0.25 J/cm^2) or vehicle (ethanol) alone. At the end of the 4-week treatment regimen, dorsal skin samples were collected for immunoflorescent staning. (Original magnification X 100)

PUVA induces homing of Tcells

Focusing on the effect of PUVA on T cell homing 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 of a 4-fold increased expression of CD62L in the case of mice treated with 0.50 J/cm^2 **(Fig. 16)**. Further, we found an up to 8-fold increase (10.90% vs. 1.36%) of CLA+CD25+CD4+ T cell numbers in the lymph nodes of K5.hTGF-β1 transgenic mice treated with 0.25 J/cm^2 compared to vehicle-treated mice (Figure 16). Furthermore, we also increased numbers of Foxp3+CD25+ T cells in the skin **(Fig. 15)**. Our results suggest extrathymic development of Tregs, which may be capable of migrating from the skin to peripheral lymph nodes or vice versa. Taken together, we therefore propose that Tregs are activated in the skin via PUVA exposure, then home to the peripheral lymph node and/or the spleen, then, after maturation, come back to the skin for a short period of time, where they secret IL-10 or other immunosuppressive cytokines. The latter activity is thought to reduce the proliferation of inflammatory T cells and, consequently, to reduce epidermal hyperplasia in K5.hTGFβ1 transgenic mice. These results help to better understand how PUVA may modulate the immune system and act in inflammatory skin diseases such as psoriasis.

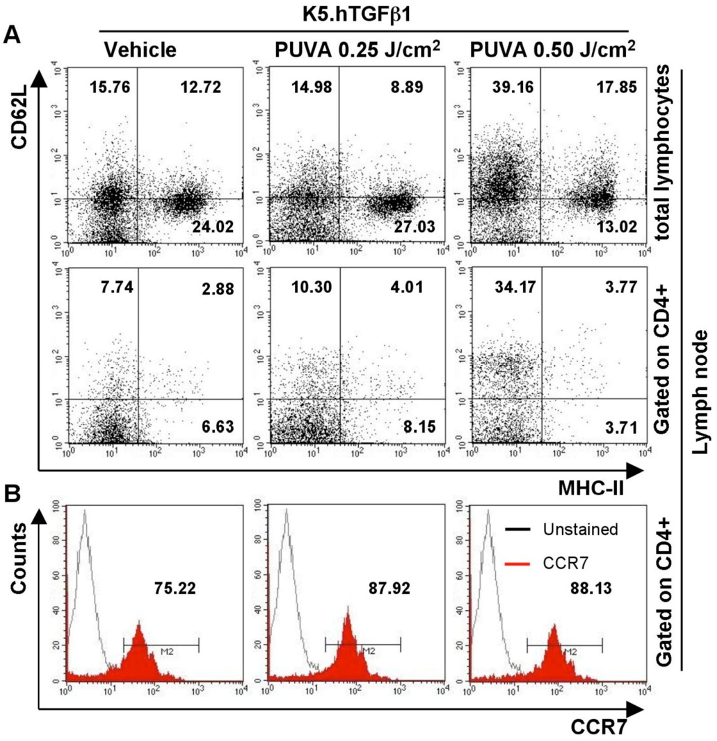

Figure 16: PUVA increases homing of CD4+ T cells

*K5.hTGF-β1 transgenic mice were treated, as described in Figure 5. At the end of the 4-week treatment regimen lymph nodes samples were collected. FACS of pooled lymph nodes of K5.hTGF-β1 mice were stained for CD4 and cell surface marker CD62L, MHC-II **(A)** and CCR7 **(B)**. Plots are either gated on CD4+ T cells or total lymphocytes. Numbers in quadrants indicate percent cells. Data shown are from one representative experiment, with n=3 pooled mice per treatment group.*

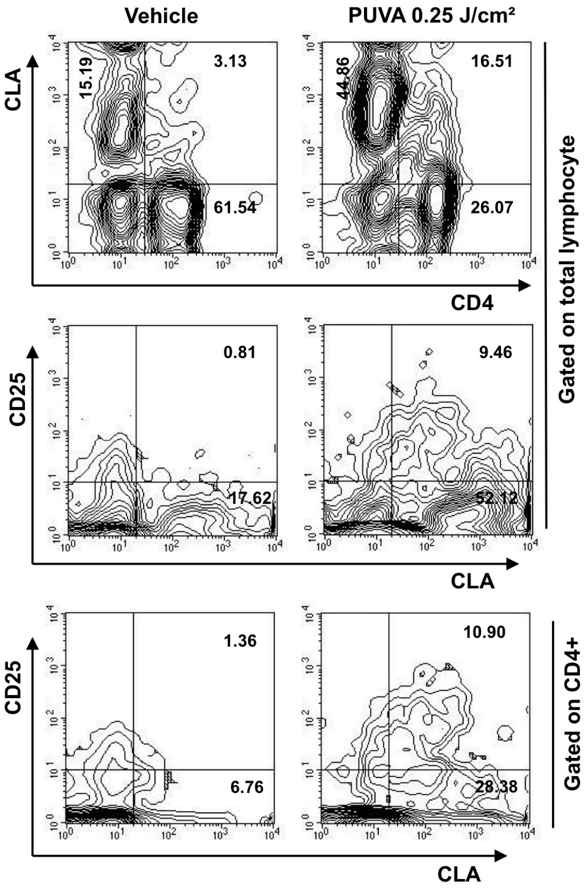

Figure 17: PUVA increases CLA+CD25+CD4+ T cell homing in K5.hTGF-β1 transgenic mice

K5.hTGF-β1 mice were topically treated on their shaved back skin twice a week for 4 weeks with either 8-methoxypsoralen 15 min before exposure to a sub-phototoxic dose of UVA (PUVA 0.25 J/cm^2) or vehicle (ethanol) alone. At the end of the 4-week treatment regimen lymph nodes samples were collected for FACS analysis. Cells were first stained with CD4 and CD25 and then after washing further stained with skin homing receptor CLA. Plots are either gated on total lymphocytes or CD4+ T cells. Numbers in quadrants indicate percent cells. Data shown are from one representative experiment, with n=3 pooled mice per treatment group.

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 2,500-fold higher than those of CD28; thus giving CTLA4 a competitive advantage over CD28 (Peggs *et al.*, 2006; Walunas *et al.*, 1996). 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 (Sakaguchi *et al.*, 2001). Moreover, blocking CTLA4 signaling has been shown to inhibit the suppressive function of UV-induced CD4+CD25+ T cells (Loser *et al.*, 2005). We therefore employed anti-CTLA4 monoclonal antibody treatment to test the functional relevance of PUVA-induced Tregs. K5.hTGF-β1 transgenic mice were treated with PUVA (twice a week four 4 weeks; 0.25 J/cm^2) **(Fig. 18A)**. When the mice were injected with anti-CTLA4 monoclonal antibody, PUVA lost its disease-suppressive activity whereas injection with an isotype control antibody did not affect the beneficial effect of PUVA. This effect was confirmed on the microscopic level (**Fig. 18 B and C**). Indeed, quantitative analysis revealed that there was no statistically significant difference in epidermal thickness (**Fig. 18C**) and inflammatory infiltrate in the dermis between anti-CTLA4 antibody-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 co-cultured with CD4+CD25- T cells, each in the absence or presence of anti-CTLA4 monoclonal antibody (**Fig. 18E**). 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 monoclonal antibody did not alter the number of CD4+CD25+Foxp3+ Tregs in the spleens of PUVA-treated transgenic mice, compared to isotype control antibody injection (**Fig. 18D**). 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. 18F**). Anti-CTLA4 (but not isotype control) antibody injection of the mice abrogated the PUVA-induced increase of IL-10 in the supernatant.

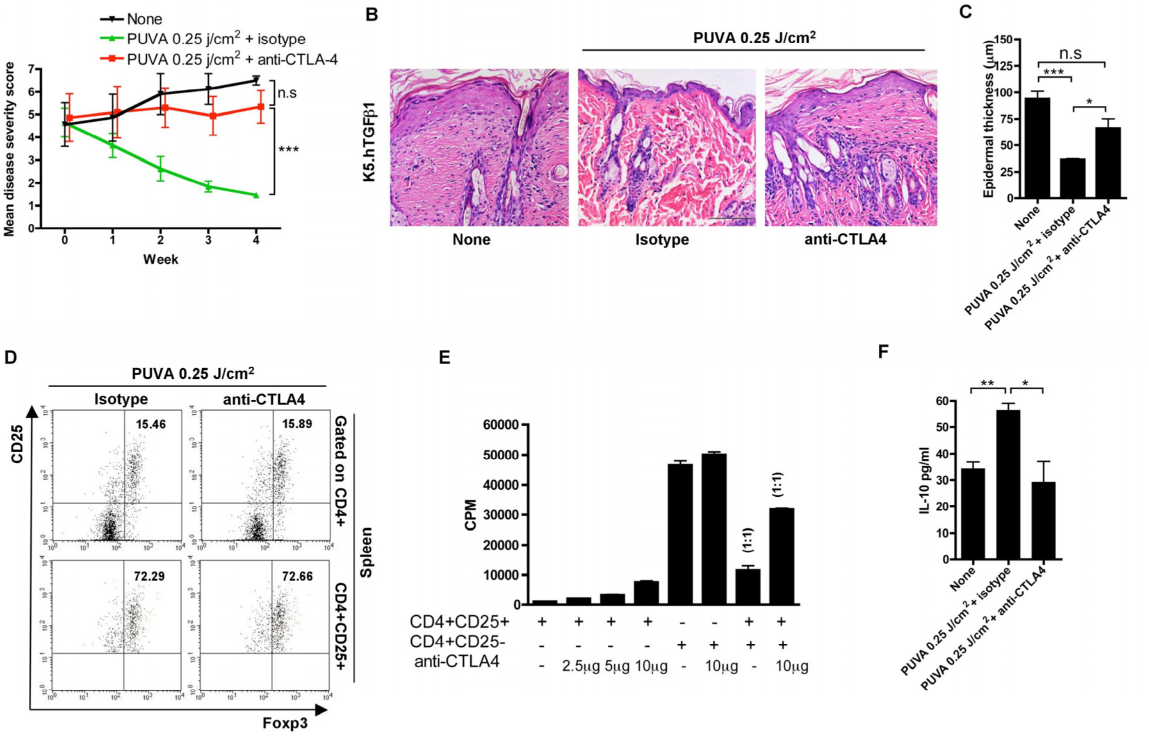

Figure 18: Anti-CTLA4 treatment abolishes PUVA-induced therapeutic effect, IL-10 upregulation, and CD4+CD25+ Treg cell function.

K5.hTGF-β1 transgenic mice were topically treated twice a week for 4 weeks with either 8-methoxypsoralen on their shaved back skin 15 min before exposure to 0.25J/cm^2 of UVA radiation or left untreated (None). Groups of mice were injected i.p. twice a week with anti-CTLA4 monoclonal antibody or an isotype control antibody immediately after PUVA exposure. ***(A)*** *Skin disease severity in the mice.* ***(B)*** *Representative images of H&E-stained sections of dorsal skin (scale bar 100 µm).* ***(C)*** *Epidermal thickness, as measured in the H&E-stained skin sections.* ***(D)*** *FACS of pooled single cell suspension from spleen, stained with monoclonal antibodies to CD4, CD25 and Foxp3. Plots are either gated on CD4+ or CD4+CD25+ T cells. Numbers in quadrants indicate percent cells.* ***(E)*** *Proliferation assay of CD4+CD25+, CD4+CD25- or the co-culture from PUVA 0.25 J/cm^2 treated mice. Each cell subset was cultured in duplicate either alone (1 x 10^5) or with an equal number (1:1) of CD4+CD25+ and CD4+CD25- cells, in the absence or presence of different concentrations of anti-CTLA4 monoclonal antibody. Cells were stimulated and analyzed, as described in materials and methods.* ***(F)*** *IL-10 level in the supernatant of the cultured splenocytes for corresponding groups as determined by ELISA. Data shown are from one representative experiment (A, C), with n=5 mice per treatment group, cells from one representative mouse of 3 mice (D), or pooled cells from 3 mice (E, F). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. n.s, not significant.*

5. DISCUSSION OF STUDY PART 1

This study addresses the molecular mechanisms of the therapeutic action of PUVA in K5.hTGF-β1 transgenic mice (**Fig. 5**), a highly ranked murine model with a skin disease of psoriasis-like character (Gudjonsson *et al.*, 2007; Han *et al.*, in press; Li *et al.*, 2004; Schon, 2008). Besides many abnormalities that also occur in human psoriasis patients, such as preponderance of the IL-23/Th17 axis (Di Cesare *et al.*, 2009a) (**Fig. 7**) and a Th1 milieu including abnormally elevated cytokines such as IFN-γ and TNF-α (**Fig. 8**), (likewise to certain other psoriasis mouse models) (Conrad *et al.*, 2007), we detected normal levels of natural Tregs (**Fig. 14**) 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 (for instance by downregulating IFN-γ expression 10-fold) (**Figs. 7 and 8**) and by simultaneously inducing an IL-10-producing CD4+CD25+Foxp3+ Treg population with disease-suppressive activity (**Fig. 14B**), that was abrogated by targeting Treg function with anti-CTLA4 (Loser *et al.*, 2005; Read *et al.*, 2006) monoclonal antibody treatment (**Fig. 18**), 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. found that depletion of T cells by crossing K5.hTGFβ1^{wt} mice with Rag1$^{-/-}$ mice delayed skin inflammation and associated epidermal hyperplasia (Han *et al.*, in press). In contrast, Michaelis et al observed that T cells had a limited role in the formation of psoriasiform skin lesions in K5.hTGF-β1 mice (Michaelis *et al.*, 2010). They found that eradication of T lymphocytes with CD4-depleting antibodies 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 pathomechanistic similarities between K5.hTGF-β1 mice and human psoriasis patients. Similar to the mouse model, i) CD25^{high} Tregs comprise similar proportions of CD4+ cells in psoriatic and normal blood, ii) psoriatic CD4+CD25^{high} Tregs cells are impaired in their inhibitory functions, fail to suppress T responder proliferation, and are anergic to polyclonal CD3/CD28 TCR stimulation in psoriasis patients, and iii) CD4+CD25^{high} Tregs from normal and untreated psoriatic patients express or produce similar amounts of IL-10 (Sugiyama *et al.*, 2005b). Another interesting aspect of the K5.hTGF-β1 mouse model concerns the homing of CD4+ T cells, and CD4+CD25+ Tregs highlighted by the increased expression of CD62L, MHC-II, CCR7 and CLA from lymph nodes of PUVA-exposed animals (**Fig. 16, 17**). Taken together we therefore speculate that Tregs and other T cells are activated in the skin via PUVA exposure, then home to the peripheral lymph node and/or the spleen, then, after maturation, come back for a short time to the skin, secret IL-10 or other immunosuppressive cytokines, and hereby may reduce the proliferation of inflammatory T cells and consequently reduce epidermal hyperplasia in K5.hTGF-β1 transgenic mice. This finding suggests migration of 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. 14D**) and its level in cells from spleens or lymph nodes (**Fig. 14A**). Foxp3 programs the development and function of CD4+CD25+ Tregs in controlling autoimmunity. Its expression is a key feature of natural Treg cells (Zheng and Rudensky, 2007), which arise during thymic development through high-affinity recognition of self antigens (Jordan *et al.*, 2001). In contrast, induced Tregs (iTregs) or so-called adaptive Tregs are Foxp3+ cells that develop extrathymically and share many phenotypic and functional characteristics of natural Tregs, but exert their regulatory activity mainly by IL-10 and TGF-β secretion (Liu *et al.*, 2003). TGF-β is crucial for the generation of induced Foxp3+ T cells (Bettelli *et al.*, 2006; Fantini *et al.*, 2004) while the presence of IL-6 inhibits the conversion of T cells into Foxp3+ Tregs and favours Th17 immunity (Korn *et al.*, 2008; Volpe *et al.*, 2008). 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 (Loser *et al.*, 2006), in which epidermal RANKL induced upon UV exposure was shown to control Treg cell numbers coupled to local and systemic immune suppression. Notably, RANKL expression is a strong inducer of vitamin D3 and vitamin D3 derivatives are successfully used to treat psoriasis (Kragballe, 1992). Moreover, UV phototherapy may augment the functional activity of Tregs in polymorphic light eruption in preseasonal photohardening. This activity is possibly associated with normalization of sun sensitivity in this presumably autoimmune-mediated condition (Wolf *et al.*, 2009). It is also worth to mention that the infusion of expanded Tregs (e.g. by photopheresis) (Maeda *et al.*, 2008) has been suggested as a therapeutic concept to control immunity in graft-versus-host-disease after allogeneic bone marrow transplantation.

We also observed in the skin of K.5hTGF-β1 transgenic mice an increased level of STAT3 (**Fig. 7A and 11**), a transcription factor that is thought to link activated keratinocytes and immunocytes required for development of psoriasis in another transgenic mouse model (Sano *et al.*, 2005), as well as orphan nuclear receptor ROR-γt that is known for orchestrating the differentiation program of proinflammatory IL-17+ T helper cells (Ivanov *et al.*, 2006). 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. 7A and B**). The importance of the IL-17 pathway in the K5.hTGF-β1 model is supported by the observation that blocking by anti-IL-17 monoclonal antibody injection stopped the progression of the skin disease, but did not clear existing lesions in the skin of the transgenic mice (**Fig. 9**). However, the anti-IL-17 antibody treatment did not affect Foxp3 levels and IL-10 mRNA levels in the skin (**Fig. 9D**). 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 (Veldhoen *et al.*, 2006). IL-17 itself has many proinflammatory effects on 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 antimicrobial peptides (Wilson *et al.*, 2007a). The significance of the IL-17 pathway in human psoriasis patients has been recently recognized (Zaba *et al.*, 2009). For instance, psoriasis lesions in human patients contain discrete populations of Th1 and Th17 cells (Lowes *et al.*, 2008), which are activated on increased production of IL-17A, IL-17F, and IL-22 mRNA (Lowes *et al.*, 2008; Zaba *et al.*, 2009). Anti-IL-17-antibody treatment is in the clinical test phase for its anti-psoriatic activity.

IL-23, a regulatory key cytokine in the differentiation and survival of Th17 cells (Volpe *et al.*, 2008), has been found up-regulated in psoriatic skin lesions (Lee *et al.*, 2004b), consistent with our observations in the K5.hTGF-β1 mouse model (**Fig. 7C and D**). Both mRNA and immunohistochemical analysis showed that IL-23p19 and IL-12p40 subunits (the latter shared by IL-12 and 23) were increased in human psoriatic plaques compared with non-involved skin; however, no difference was observed in human subjects for the IL-12p35 subunit (Lee *et al.*, 2004b), suggesting that IL-23 is playing a more dominant role than IL-12 in psoriasis. This is absolutely in line with the results presented here, showing that IL-12p40 and IL-23p19 expression and/or protein was increased (and responded to PUVA) (**Fig. 7A-D**) but IL-12p35 expression was not increased in the diseased skin of the transgenic mice (**Fig. 7A**). At first glance, these results seem to be contradictory to the recent study by Fitch et al (Fitch *et al.*, 2009) 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 lesional IL-4 and high-serum IgE levels, contrasting to the characteristics of the K5.hTGF-β1 mice originally described by Li et al (Li *et al.*, 2004) and confirmed in our study. The effect of PUVA on IL-23 levels in the skin is consistent with that of other antipsoriatic treaments such as UVB 311 nm therapy (Piskin *et al.*, 2004), cyclosporin A, and biological agents modulating this cytokine (Di Cesare *et al.*, 2009a). Th22 cells represent another distinct human T cell subset uniquely able to regulate epidermal responses in inflammatory skin disease ((Eyerich *et al.*, 2009; Kagami *et al.*, 2009)), however, the effect of antipsoriatic 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 (Wolf *et al.*, 2006). 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 (Asadullah *et al.*, 2003) and IL-10 (and likewise IL-4) treatment has been shown to exhibit clinical efficacy in psoriasis (Ghoreschi *et al.*, 2003b; Reich *et al.*, 2001). It was, therefore, coherent 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. 8**), being crucial for the formation of iTregs with antipsoriatic activity (**Figs. 14A,E and 18C**).

However, PUVA downregulated several other cytokines and chemokines such as IL-1a, IL-6, IL-9, IP-10, MCP1, (keratinocyte chemoattractant) KC, RANTES, G-CSF, MCP (monocyte chemoattractant protein) and GM-CSF, identifying them as potential therapeutic targets in autoimmune diseases such as psoriasis and other conditions, which are responsive to PUVA (Wackernagel *et al.*, 2006). Interestingly, the higher PUVA dose 0.50 J/cm^2 was more effective in increasing or reducing the levels of most cyokines (**Figs. 7 and 8**), whereas the lower PUVA dose 0.25 J/cm^2 was more effective in clearing the psoriatic lesions in the mice (**Fig. 5B**). However, this seemingly paradox observation is consistent with the clinical knowledge that sub-erythemal 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, (Chaudhari *et al.*, 2001) IL-12/23 (Krueger *et al.*, 2007) or IL-17. PUVA and other forms of phototherapy may act as a type of natural anti/pro-cytokine therapy, and, therefore, the combination with synthetic biologics may actually represent a novel synergistic multi-biologic 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 antibody 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 antibody treatment prevented disease progression whereas PUVA improved the skin status of the transgenic mice, an effect that was abrogated by anti-CTLA4 antibody 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 (Stern, 2007; Wolf *et al.*, 2004). Interestingly, upon chronic UV exposure, Tregs were found to be involved in skin cancer formation in mice (Loser *et al.*, 2005) and are targeted in human cancer immunotherapy (Peggs *et al.*, 2006). On the other hand, IL-23 and IL-17 are increased in human tumors and have been implicated in causing cancer (Tato and O'Shea, 2006).

6. RESULTS OF STUDY PART 2

The results of study part 2 have been submitted for publication in 2010:

Tej Pratap Singh, Barbara Huettner, Harald Koefeler, Gerlinde Mayer, Isabella Bambach, Katrin Wallbrecht, Michael P. Schön, Peter Wolf

Platelet-activating factor blockade inhibits Th17 pathway and suppresses psoriasis-like skin disease in K5.hTGF-beta1 transgenic mice

PAF receptor blockade prevents whereas PAF accelerates progression of skin disease in K5.hTGF-β1 transgenic mice

In order to study the role of PAF and its receptor in the pathogenesis of psoriasis-like skin disease in K5.hTGF-β1 transgenic mouse model we used the PAF antagonist PCA-4248. This antagonist has been previously shown not only to inhibit UV-induced immunological alterations (Matsumura *et al.*, 2006; Walterscheid *et al.*, 2002; Wolf *et al.*, 2006) but also non-melanoma skin cancer induction and progression and UV-induced skin damage in hairless mice (Sreevidya *et al.*, 2008), as well as experimental human melanoma lung metastasis in nude mice (Melnikova and Bar-Eli, 2007). We injected wild type or K5.hTGF-β1 transgenic mice either with PAF antagonist PCA-4248 or PBS as vehicle control twice weekly for four weeks. We found that treatment with PCA-4248 blocked the psoriatic disease progression in K5.hTGF-β1 transgenic mice compared to vehicle-injected control littermates **(Fig. 19, A and B)**. The stop of progression of skin disease in the transgenic mice was paralleled by the reduced histological alterations that we observed in the PCA-4248-treated K5.hTGF-β1 transgenic mice compared to vehicle-injected control littermates **(Fig. 19C)**. Whereas vehicle-treated K5.hTGF-β1 transgenic mice exhibited a hyperplastic epidermis of approximately four cell layers and a thickness of more than 60µm and a dermis with a superficial mixed inflammatory infiltrate consisting of mononuclear cells and neutrophils, CD68+ cells and CD3+T cells **(Fig. 19D and 20)**, such skin alterations were significantly diminished by approximately 20 to 55% in the skin of PCA-4248-injected transgenic mice after 4 weeks of treatment **(Fig. 19D)**. PCA-4248 injection particularly reduced the number of neutrophils (from 100.0±3.2 to 69.2±6.8), CD68+ cells (27.8±3.2 to 17.9±1.4) and CD3+T cells (42.4±1.1 to 26.7±2.6) in the dermis of the transgenic mice **(Fig. 20)**. In contrast, we found that injecting PAF into the skin of the transgenic mice led to inflammation and accelerated the manifestation of the psoriatic phenotype by a local effect, as observed on the macroscopic level and confirmed on the microscopic level by density of skin infiltration and epidermal hyperplasia. The effects of PAF in accelerating the disease phenotype were more prominent after 72 hours compared to 24 hours after single intracutaneous injection **(Table 1).**

Table 1. PAF accelerates the skin disease phenotype of K5.hTGF-β1 transgenic mice.

Histological parameter	**Time point / Treatment**							
	24h				**72h**			
	WT		K5.hTGF-β1		WT		K5.hTGF-β1	
	Vehicle	PAF	Vehicle	PAF	Vehicle	PAF	Vehicle	PAF
Skin thickness (μm)	15.1±1.3	18.7±2.0	24.8±2.1	38.7±4.3*	21.7±2.2	26.8±5.5	31.5±3.7	54.4±2.9*
No. of epidermal layers	1.8±0.3	2.0±0.3	3.0±0.2	3.4±0.1	1.6±0.1	2.0±0.2	3.0±0.3	4.0±0.3*
Total dermal cell infiltrate score	0.3±0.1	0.3±0.1	1.5±0.1	2.3±0.2**	0.3±0.1	0.4±0.2	1.7±0.1	2.5±0.2**
No. of neutrophils per 200x field	8.5±1.4	13.8±1.8	55.9±6.0	76.8±9.4	7.8±0.9	18.3±1.9**	59.8±4.9	93.5±5.0**

Mice were injected intracutaneously with PAF (100 ng) or with vehicle control. Biopsies were taken at injection sites at different time points after injection (24h and 72h) for histological evaluation, as defined in materials and methods. *, $P<0.05$; **, $P<0.01$; (unpaired student t-test) comparing the respective PAF vs. vehicle treatment values (n=4-8 per treatment and time point).

In addition to being a PAF receptor antagonist, PCA-4248 may also act as a 5-HT receptor antagonist. We therefore looked for the effect of the 5-HT receptor antagonist ketanserin tartrate on the psoriatic phenotype in K5.hTGF-β1 transgenic mice. However, we found that blocking 5-HT receptor with ketanserin tartrate did not affect macroscopic skin phenotype (**Fig. 21A**) as well as microsopic skin alterations in the transgenic mice (data not shown). Furthermore, intracutaneous injection of 5-HT did not accelerate the psoriatic disease phenotype of the transgenic mice (**Fig. 21B**). These findings indicated that the effect of PCA-4248 in blocking PAF was specific and 5-HT did not play a (major) role in psoriatic skin disease pathology of K5.hTGF-β1 transgenic mice.

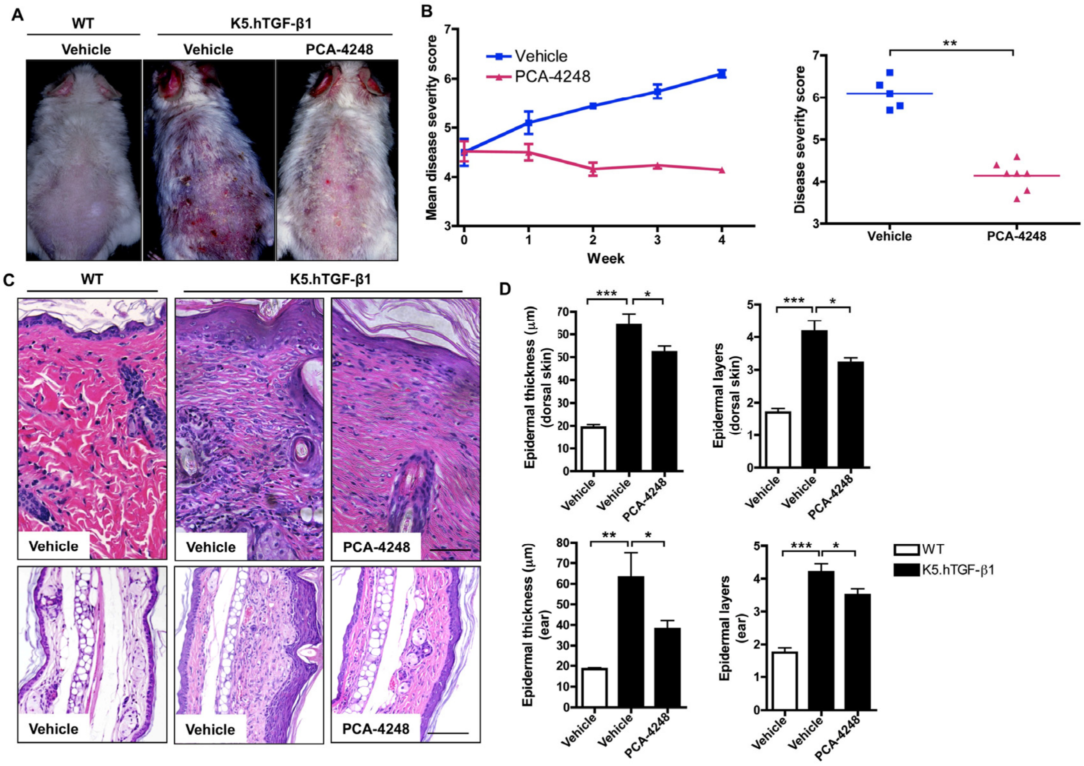

Figure 19: PCA-4248 blocks macroscopic and microscopic psoriatic skin disease progression in K5.hTGF-β1 transgenic mice

WT or K5.hTGF-β1 transgenic mice were injected i.p. twice weekly for 4 weeks with PCA-4248 (10mg/kg) or its vehicle. ***(A)*** *Macroscopic presentation of WT or K5.hTGF-β1 transgenic mice treated either with PCA-4248 or vehicle.* ***(B)*** *A specific score (ranging from 0 to a maximum of 9) was used to monitor macroscopic disease severity in the mice. Results are represented either as mean disease score (left) during the 4-week treatment regimen or disease severity score at week 4 (right).* ***(C)*** *Representative images of H&E-stained sections from dorsal skin or ear of a mouse of the different treatment groups (scale bar 100 µm). (D) Evaluation of histological features including epidermal thickness and epidermal layers. Data shown are from one representative experiment, with n=5-10 per treatment group. *, p≤0.05; **, p≤0.01; ***, p≤0.001.*

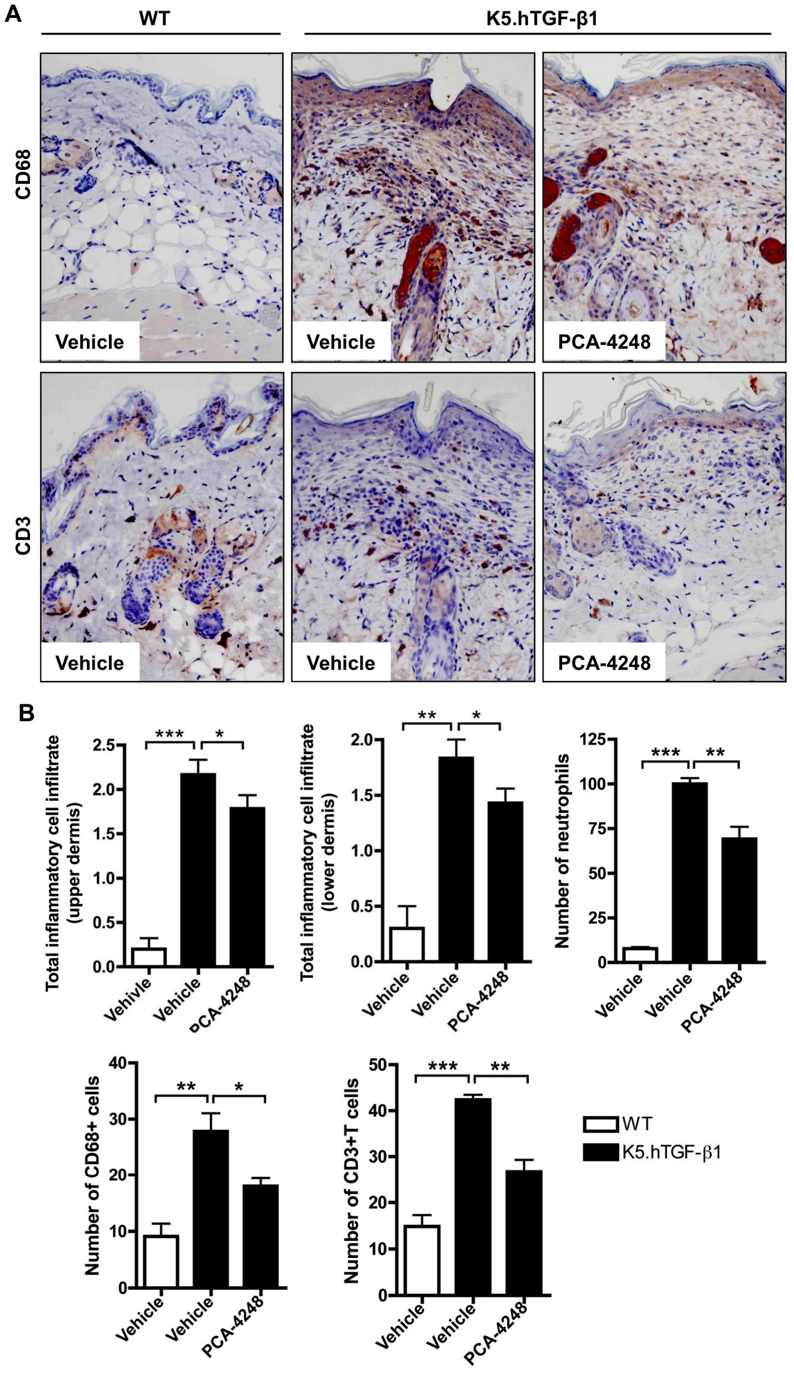

Figure 20: PCA-4248 decreases accumulation of different inflammatory cells in the dermis of K5.hTGF-β1 transgenic mice

WT or K5.hTGF-β1 transgenic mice were injected i.p. twice weekly for 4 weeks with PCA-4248 (10mg/kg) or its vehicle. Dorsal skins were collected at the end of study from different groups for assessment of different inflammatory cell infiltrate. (A) Representative images of immunohistochemically stained sections of CD68, CD3 and mast cells from dorsal skin of a mouse of the different treatment groups (scale bar 100 µm). (B) Evaluation of histological or immunohistochemical sections, including total inflammatory cell infiltrate (0, no; 1, mild; 2, moderate; 3, severe) in the upper or lower *dermis; number of neutrophils***,** *CD68+ cells (monocytes/macrophages), CD3+T cells and mast cells. Data shown are from one representative experiment, with n=3-10 per treatment group. *, p≤0.05; **, p≤0.01; ***, p≤0.001.*

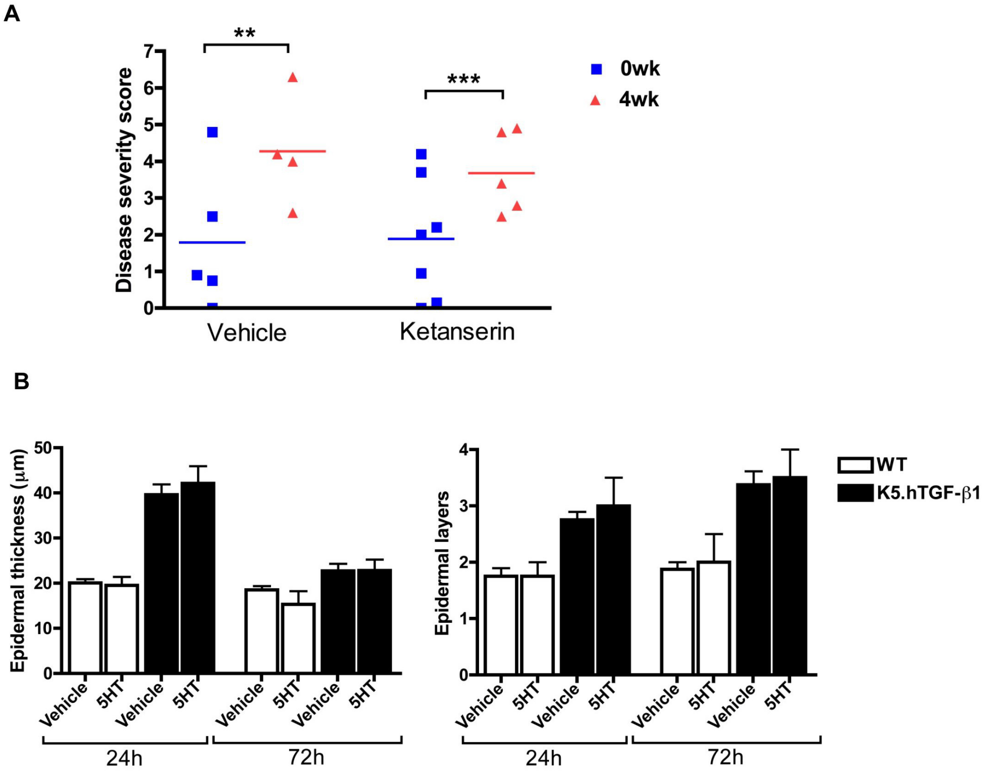

Figure 21: ***(A)*** *K5.hTGF-β1 transgenic mice were injected i.p. twice weekly for 4 weeks with serotonin receptor antagonist ketanserin tartrate (10mg/kg) or its vehicle. A specific score (ranging from 0 to a maximum of 9) was used to monitor macroscopic disease severity score, as described in material and methods. Note the disease score significantly increased over the 4-week treatment period in both treatment groups. There was no significant difference in the treatment score at week 0 and week 4 between the two treatment groups.* ***(B)*** *Mice were injected intradermally on abdominal skin with 5-HT (5 µg) or with vehicle control. Biopsies were taken at injection sites at different time points after injection (24h and 72h) for histological evaluation, as defined in materials and methods (n=2-5 per treatment and time point). Note there was no significant difference in epidermal thickness and epidermal layers between vehicle and 5-HT treatment at 24h and 72 h.*

Both PAF receptor blockade with receptor antagonist PCA-4248 and PUVA normalize the PAF level in the skin of K5.hTGF-β1 transgenic mice

We had hypothesized that PAF plays a role in the pathogenesis of the psoriasis-like skin disease in K5.hTGF-β1 transgenic mice. In order to determine PAF levels and study the potential effect of PAF receptor blockade by an autocrine loop (Maggi *et al.*, 1994; Peri *et al.*, 2003) on PAF itself we measured PAF levels directly in the skin of the mice by mass spectrometry. We found that vehicle-injected K5.hTGF-β1 transgenic mice had significantly increased PAF levels (0.814±0.16 ng/100 mg) in the skin compared to wild type littermates (0.10±0.10 ng/100 mg) at the end of the 4-week treatment protocol **(Fig. 24A)**. PAF receptor blockade with the antagonist PCA-4248 (but not vehicle control) reduced the PAF levels in the skin of K5.hTGF-β1 transgenic mice to 0.114±0.02 ng/100mg, a level similar than observed in wild type control mice **(Fig. 24, A and B)**. We have previously demonstrated that PUVA given at sub- (0.25 J/cm^2) or near-phototoxic (0.50 J/cm^2) doses were effective in clearing psoriasis-like skin symptoms in K5.hTGF-β1 transgenic mice. We therefore were interested to determine the effect of PUVA on PAF and found that both PUVA doses, similar to PCA-4248, reduced) the PAF levels in the skin of K5.hTGF-β1 transgenic mice to 0.166±0.06 and 0.286±0.10 at 0.25 J/cm^2 and 0.50 J/cm^2, respectively **(Fig. 24A)**. The observation that the PAF-depressing effect of the lower PUVA dosage of 0.25 J/cm^2 was greater than that of the higher dose of 0.5 J/cm^2, is consistent with the potential role of PAF in the pathogenesis of the skin disease in K5.hTGF-β1 transgenic mice, in which the lower, subphototoxic PUVA dose was also slightly more effective in reducing the psoriasis-like skin symptoms in the mice (Singh *et al.*, 2010). This also goes in line with the clinical knowledge that under certain circumstances too high PUVA doses can trigger or aggravate psoriasis by the so called Koebner phenomenon (Wolf *et al.*, 2006). Moreover, we have previously provided evidence that exposure of normal mouse skin may lead to the production of PAF and/or PAF-like molecules with immunosuppressive properties, depending on the PUVA dosage used (Wolf *et al.*, 2006).

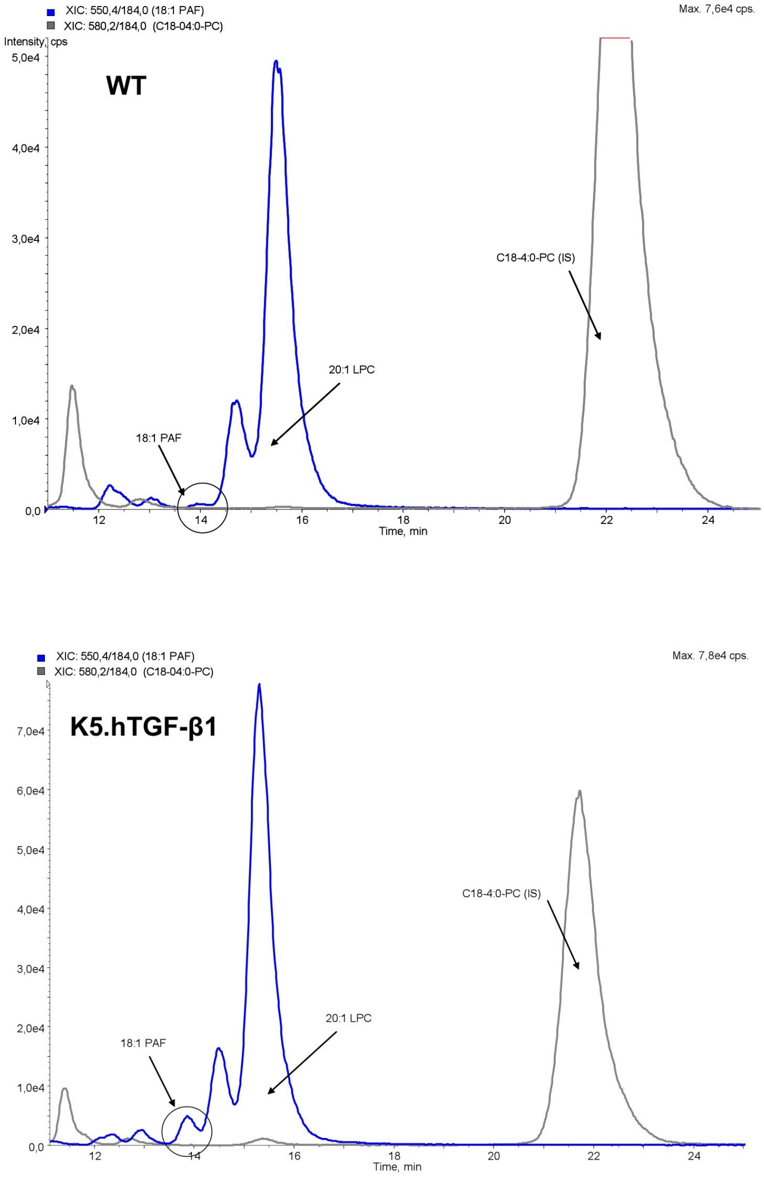

Figure 22: *Representative single reaction monitoring chromatogram from LC-MS/MS analysis of a sample from an individual wild type mouse (WT, upper graphic) or K5.hTGFβ1 transgenic mouse (lower graphic). The extracted mass traces reflect PAF 18:1, lysophosphatidylcholine 20:1 and the internal standard C18:0-4:0 PC. (Figure 22 and 24B provided by Harald Koefeler and Barbara Huettner; Center for Medical Research, Medical University of Graz, Austria)*

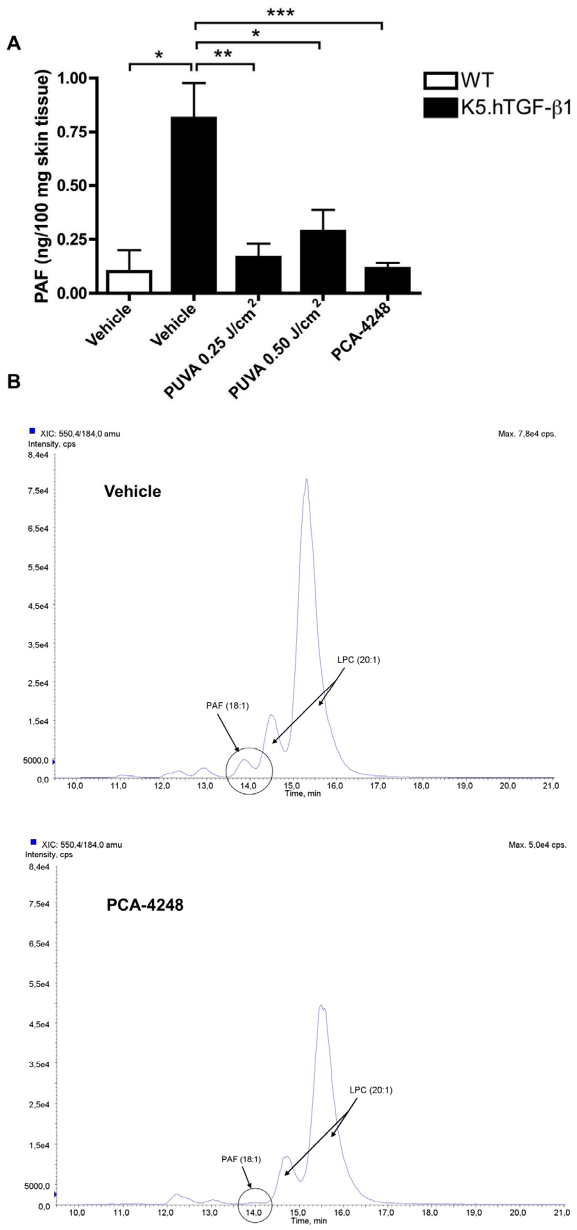

Figure 24: Both PCA-4248 and PUVA decrease the PAF level in the skin of K5.hTGFβ1 transgenic mice

(A) *PAF levels were analyzed in skin samples from WT or K5.hTGFβ1 transgenic mice injected either with PCA-4248 or its vehicle or transgenic mice treated with a sub- (0.25 J/cm^2) or near-phototoxic (0.50 J/cm^2) dose of PUVA. PCA-4248 as well as PUVA significantly decreased the level of PAF in dorsal skin of the transgenic mice, as detected by mass spectrometry. Data shown are pooled from two independent experiments, with n=3-7 per treatment group. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.* ***(B)*** *Representative histogram of the result of mass spectrometric analysis of a sample from an individual K5.hTGFβ1 transgenic mouse injected either with vehicle or PCA-4248. PAF (18:1), peak of PAF; LPC (20:1) peak of lysophosphatidylcholine.*

PAF receptor blockade downregulates STAT3 and Th17 cytokine pathway but upregulates IL-10 in K5.hTGF-β1 transgenic mice

We found that blockade of PAF receptor with the PAF receptor antagonist PCA-4248 depressed the abnormally elevated protein levels of IL-6, IL-12, IL-17A , and IL-23 by 36, 52 and 76%, respectively, and increased IL-10 by 44%(1.44 fold up) in the serum of K5.hTGF-β1 transgenic mice **(Figure 25A)**. However, it did not significantly affect or reduce the serum levels of IL-2, IL-4, IL-5, IL-13, IFN-γ and TNF-α. We also found that PAF receptor blockade with PCA-4248 significantly reduced the mRNA expression of STAT3, IL-17A, IL-17F, IL-12A and IL-6 and upregulated the mRNA expression of COX2 and IL-10 in the dorsal skin of K5.hTGF-β1 transgenic mice compared to vehicle-injected mice **(Figure 25D)**. Immunofluorescent staining of dorsal mouse skin revealed that PCA-4248 injection reduced the number and intensity of STAT3 positive cells particularly in the epidermis and increased the number of IL-10-positive cells in the dermis of transgenic mice **(Figure 25, B and C**). Weak IL-10 staining was also present in the basal layers of the epidermis but there was no difference among the treatment groups.

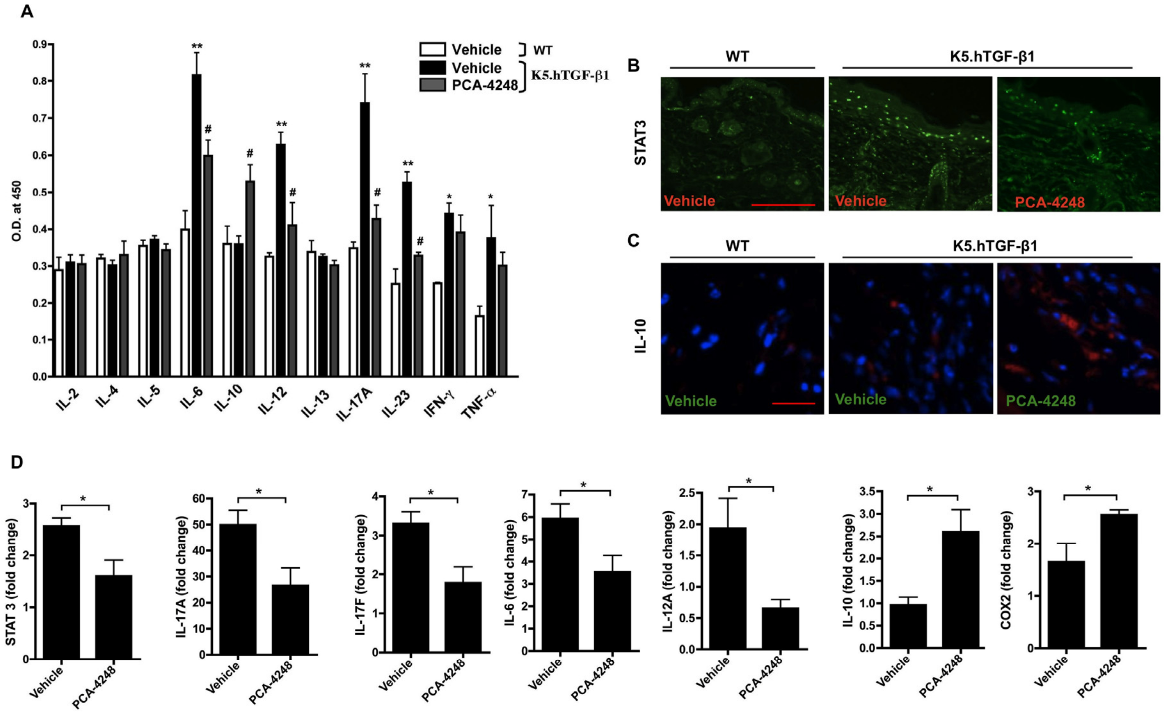

Figure 25: Effect of PCA-4248 on mRNA and/or protein level of Th1, Th2, and Th17-related cytokines, and transcription factor STAT3 and COX2 in K5.hTGFβ1 transgenic mice

WT or K5.hTGF-β1 transgenic mice were injected i.p. twice weekly for 4 weeks with PCA-4248 (10mg/kg) or its vehicle. ***(A)*** *Serum was collected at the end of the 4-week treatment regimen either from vehicle-treated WT or vehicle- or PCA-4248 treated K5.hTGFβ1 transgenic mice. A multi-cytokine ELISA was performed to analyze the relative levels of IL2, IL4, IL5, IL6, IL10, IL12, IL13, IL17A, IL23, IFNγ, and TNFα. Data shown are from one representative experiment, with n=3-4 per treatment group. *p≤0.05; **p≤0.01, vehicle-treated K5.hTGFβ1 vs. vehicle-treated WT; #, p≤0.05, PCA-4248-treated vs. vehicle-treated K5.hTGFβ1 mice.* ***(B)*** *Immunofluorescent staining of STAT3 and IL-10* (blue; DAPI, red; IL-10) ***(C)*** *protein in dorsal skin (scale bar 200 and 100 μm, respectively).* ***(D)*** *Quantitative RT-PCR was performed at the end of the 4-week treatment regimen either from the vehicle treated WT or vehicle or PCA-4248 treated K5.hTGFβ1 transgenic mice with RNA isolated from individual mouse dorsal skin for transcription factors STAT3 and transcripts encoding IL-17A, IL-17F, IL-12A, IL-6, IL-10, and COX2. Data shown are from one representative experiment, with n=5-7 per treatment group. *, p≤0.05.*

PAF receptor blockade not only stopped the disease progression on the macroscopic and microscopic level **(Fig. 19)** but also reduced the PAF level in the skin **(Fig. 24).** Although the exact mechanism, by which PAF receptor blockade did reduce PAF levels, remains to be determined, the most likely explanation is simple interruption of the known PAF autocrine loop (Maggi *et al.*, 1994; Peri *et al.*, 2003) and/or by interfering with leukocyte (e.g PMN) infiltration into the skin; the PAF receptor antagonist is inhibiting PAF formation, especially since these cells produce large amounts of this mediator.

We also found that blockade of the PAF receptor with the antagonist PCA-4248 depressed the abnormally elevated protein levels of IL-6, IL-12, IL-17A, and IL-23, and increased IL-10 in the serum **(Fig. 25A)** and significantly reduced the mRNA expression of STAT3, IL-17A, IL-17F, IL-12A and IL-6 in the dorsal skin of the transgenic mice **(Fig. 25D)**. This is important because recent studies described the significance of the Th17 pathway and its involvement in model systems of autoimmunity including psoriasis (Di Cesare *et al.*, 2009a). The development and maintenance of psoriatic lesions have been linked with the secretion of cytokines and chemokines including Th17-related cytokines such as IL-17A, IL-17F, IL-23, IL-12 and IL-6 (Lowes *et al.*, 2007b). For instance, Teunissen *et al.* (Teunissen *et al.*, 1998a) showed detectable levels of IL-17 mRNA in lesional psoriatic skin, but not in non-lesional skin, and showed that CD4+ and CD8+ clones derived from lesional psoriatic skin were able to produce IL-17 after stimulation with CD3/CD28 antibodies. The importance of the IL-17 pathway in the K5.hTGF-β1 mice is also supported by the observation that blocking by injection with anti-IL-17 monoclonal antibody does also stop the progression of the psoriatic skin disease in the model **(Fig. 9)**. At first sight, this seems to contradict the study by Fitch *et al.* (Fitch *et al.*, 2009) suggesting that the inflammatory skin disease in their K5.hTGF-β1 mice did not depend on the IL-23/Th17 pathway. However, the animals they used exhibited eosinophilic skin infiltration and highly elevated lesional IL-4 and high-serum IgE levels (after years of breeding presumably due to changes in transgene expression), contrasting to the characteristics of the K5.hTGF-β1 mice originally described by Li et al (Li *et al.*, 2004). The latter findings were confirmed in our study, in which the mice had normal expression of Th2 cytokines, including IL-4 **(Fig. 25A)**.

We also found that blockade of the PAF receptor with PCA-4248 significantly reduced neutrophil infiltration in the dermis of the transgenic mice **(Fig. 19D)**. This effect may have been mediated via negative regulation of leukotactic cytokines such as functional murine IL-8 homologues CXCL1/KC, CXCL2/MIP-2 (Countryman *et al.*, 2000; Pei *et al.*, 1998). This is of clinical relevance because the abundance of PAF, e.g. induced by intradermal injection into human skin, induces local swelling, wheal and flare responses, intravascular congestion and neutrophil accumulation with the development of a cellular infiltrate, primarily composed of lymphocytes and histiocytes (Archer *et al.*, 1985; Edwards and Constantinescu, 2009). This goes in line with our finding that PAF injection into the skin accelerated the psoriatic phenotype of the transgenic mice **(Table 1)**. It is also important to mention that there is evidence for a cross-talk between human neutrophils and Th17 cells by the way that IL-17A and IL-17F, two of several cytokines produced by T helper 17 cells, are able to indirectly induce the recruitment of neutrophils (Pelletier *et al.*, 2009). As a consequence, PCA-4248 treatment may also have diminished the accumulation of CD68+ cells and CD3+T cells in dermis of transgenic mice **(Fig. 20)**.

In addition, we noticed that blockade of the PAF receptor activated the transcription of COX2 and the transcription and expression of IL-10 in the skin **(Fig. 25, C and D)** and its release in the serum **(Fig. 25A)**. The transcription of COX2 after PAF receptor blockade may reflect the involvement of this enzyme in an anti-psoriatic gene transcription process including IL-10 establishment in K5.hTGF-β1 transgenic mice. This finding goes in line with reports by others (Shreedhar *et al.*, 1998), who concluded that COX2-mediated PGE2 up-regulates IL-10, which down-regulates IL-12 production (Harizi *et al.*, 2002). At first glance this contrasts the results of previous studies, in which PAF was suggested to induce COX2 (Pei *et al.*, 1998; Ramos *et al.*, 2004; Walterscheid *et al.*, 2002). However, the differential outcome of COX2 regulation by PAF may be explained by differences between the cytokine networks under pathologic conditions occuring in a disease model such as K5.hTGF-β1 transgenic mice compared to physiologic conditions in normal cell culture (Pei *et al.*, 1998) or healthy mice. (Suggestion: In any case, these findings suggest that the respective net regulatory interactions in vivo have to be interpreted with caution and may be more complex than can be discerned from a single model.)

The observation that IL-10 induced by PAF blockade may be responsible for inhibiting the progression of the psoriatic phenotype in K5.hTGF-β1 transgenic mice is intriguing because recombinant IL-10 has been shown to be clinically effective in the treatment of psoriasis in humans (Reich *et al.*, 1998; Reich *et al.*, 2001), and is one of the main mediators of the effects of PUVA (Wolf *et al.*, 2006), another highly effective treatment modality of psoriasis (Legat *et al.*, 2004).

Importantly, the functional relevance and significance of PAF receptor blockade and subsequent reduction of PAF in the K5.hTGF-β1 transgenic model is supported by the findings of a previous study **(Fig. 5, 6 and 7),** in which treatment over a 4-week period with the very same doses of PUVA than used in this study led to improvement of the psoriatic phenotype of the transgenic mice, accompanied by normalization of abnormally elevated cytokine levels in the skin and the serum, including the Th1 and Th17 pathway and induction of IL-10. Similar to PUVA treatment of K.5hTGF-β1 transgenic mice we also observed upon PCA-4248 injection a decreased skin level of STAT3, a transcription factor that directly binds to the IL-17A and IL-17F promoters (Chen *et al.*, 2006), analogous to other T helper cell subsets initiates Th17 commitment (Korn *et al.*, 2007; Mathur *et al.*, 2007), and links activated keratinocytes and immunocytes required for development of psoriasis in another transgenic mouse model (Sano *et al.*, 2005). Furthermore, STAT3 has been shown to be an important regulator for other inflammatory cytokines such as IL-6 (Pflegerl *et al.*, 2009) and IL-12 (de Paus *et al.*, 2008), both downregulated by PAF blockade in this study **(Fig. 25)**. In this context it has also been previously shown that STAT1 and STAT3 translocated to the nucleus following PAF stimulation (Lukashova *et al.*, 2001) and there was attenuation of STAT-3 phosphorylation by PAF receptor antagonists (Deo *et al.*, 2002; Deo *et al.*, 2004).

Taken together, this study demonstrates that binding of PAF to its receptor is essential for psoriatic disease progression in the K5.hTGF-β1 transgenic mice and that PAF downregulation and subsequent modulation of transcription factors such as STAT3 and downstream cytokine networks by a PAF receptor antagonist such as PCA-4248 or PUVA treatment has disease suppressive activity. This implies that PAF blockade may be an option for the clinical development of antipsoriatic treatment. This particularly concerns less the chronic plaque-type (Elbers *et al.*, 1994) but more the inflammatory variants of the disease such as localized pustular psoriasis (i.e. palmoplantar psoriasis of Barber-Königsbeck or acrodermatitis continua Hallopeau) (Ahmad and Rogers, 2007; Kasche *et al.*, 2007; Thielen *et al.*, 2008) and/or generalized pustular psoriasis (von Zumbusch type) (Trent and Kerdel, 2004), which are characterized by massive neutrophilic accumulation with formation of microabscesses in the skin. The complications of the later condition can be fatal, usually as a result of the protean manifestations of severe systemic disease, congestive heart failure, and intercurrent infections. There is an urgent need for new treatments for the latter conditions since antipsoriatic drugs such as the biologics (including anti-TNF-alpha or anti- IL12/23 antibody) (Nestle *et al.*, 2009b) have only been used in single cases but have not yet been studied in controlled clinical trials and are not approved in these psoriasis variants (Ahmad and Rogers, 2007; Kasche *et al.*, 2007; Thielen *et al.*, 2008). In addition, the blockade of PAF may be advantageous compared to other anti-psoriatic strategies because it may comprise additional anti-carcinogenic efficacy (Sreevidya *et al.*, 2008) being of particular value for psoriasis patients prone to skin cancer due to previous treatment (Kreimer-Erlacher *et al.*, 2003). Moreover, interference with PAF and downstream IL-17 pathway may also be attractive to prevent comorbidities in psoriasis, especially metabolic syndrome and cardiovascular disease (Boehncke *et al.*, 2007; Gottlieb and Dann, 2009; Nijsten and Wakkee, 2009). Indeed, the IL-23/IL-17 axis is stimulated in obesity (Sumarac-Dumanovic *et al.*, 2009) and can accelerate atherosclerosis (Pejnovic *et al.*, 2009). The blockade of PAF may not only offer the opportunity to downregulate IL-17 and consequently diminish psoriatic skin manifestations but also prevent platelet aggregation (Rasheed and Saeed, 2004) and vasculopathy (Crawford *et al.*, 1999).

Generation and genotyping of K5.hTGF-β1 mice

K5.hTGF-β1 transgenic mice expressing human wild type TGF-β1 in the epidermis were generated as previously described (Li *et al.*, 2004). Mice were crossed back to the Hsd:ICR(CD-1R) background. Mice were genotyped on tail DNA by PCR analysis using primers specific for TGF-β1^{wt} transgene forward: 5`-TCTGCTGAGGAGGCTCAAGTT -3`, reverse: 5`-ACCTCGGCGGCCGGTAG-3`. All animals were maintained with alternating 12-h light and dark cycles and controlled temperature and humidity in facilities approved by the Austrian Government. Water and food was provided ad libitum. All animal procedures were approved by the Austrian Government, Federal Ministry for Science and Research, through protocol no. BMBWK-66.010/0034-BrGT/2006 and 66.010/79-C/GT/2007. Mice were 12 to 16 week old at the start of the experiment and were age- and sex-matched within each experiment.

Antibodies

The following antibodies used for FACS were from eBiosciences: Alexa Fluor conjugated anti-IL-17A (clone, eBio17B7), phycoerythrin conjugated anti-IL-17F (clone, eBio18F10), phycoerythrin conjugated anti-IFN-γ (clone, XMG1.2), fluorescein isothiocyanate conjugated anti-CD4 (clone, RM4-5), phycoerythrin conjugated anti-CD25 (clone, PC 61.5), phycoerythrin conjugated anti-CD62L (clone, MEL-14), allophycocyanin conjugated anti-MHC-II (clone, M5/114.15.2), phycoerythrin conjugated anti-CCR7 (clone, 4B12), allophycocyanin conjugated anti-Foxp3 (clone, FJK-16s). Phycoerythrin conjugated anti-IL-10 antibody (clone, JES5-16E3) was from BD Pharmingen. CLA (clone, HECA-452) from BD biosciences, San Jose and alexa fluor 647 conjugated goat anti-rat IgG secondary antibody from Invitrogen GmbH.

8-methoxypsoralen plus UVA (PUVA) treatment

The backs of the mice were shaved 1 day before PUVA treatment with an electric clipper. The remaining hair was then removed by the application of a commercial depilation cream for 3 to 5 minutes (Depilan sensitive) and subsequent cleaning with water. Groups of mice were painted on their backs with 200 µl of 8-MOP (Sigma-Aldrich) in ethanol (at a concentration of 0.1 mg/ml), vehicle (95% ethanol) or were left untreated. The mice were then kept for 15 minutes in individual compartments of standard cages to allow penetration of 8-MOP. UVA irradiation was performed using a Waldmann UV236A irradiation system carrying 2 fluorescent PL 36W/09-PUVA light tubes (emission range, 315 to 400 nm; peak, 365 nm; Waldmann Medizintechnik) at a mean irradiance of 8.55 mW/cm^2 at a distance of 15 cm from the dorsal skin of the mouse to the glass cover of irradiation system positioned up-side 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 controls, as previously described and used in other strains of mice (Wolf *et al.*, 2006). Those studies revealed that maximum skin swelling was present in the mice 48 hours after single PUVA exposures and the minimal phototoxic dose was 0.5 J/cm^2 for the dorsal skin of wild type mice and 1.0 J/cm^2 for the diseased dorsal skin of the K5.hTGF-β1 transgenic mice. For repeated treatment of the mice, PUVA was given on a subphototoxic level at a dose of 0.25 J/cm^2 (mean exposure time, 29 sec) or near-phototoxic level of 0.50 J/cm^2 (mean exposure time, 59 sec) UVA twice a week (Monday and Thursday or Tuesday and Friday) for 4 weeks.

Anti-antibody treatment

Anti-mouse IL-17 rat monoclonal antibody (clone, 50104, R&D Systems) was used for in vivo intraperitoneal (i.p.) injection of groups of mice to neutralize IL-17 bioactivity. To study the functional significance of Tregs, mice were injected i.p. immediately after PUVA exposure (0.25 J/cm^2) with 60 μg of monoclonal anti-CTLA4 rat antibody (clone, 63828, R&D Systems). As control, an isotype matched antibody (rat IgG antibody, R&D Systems) was given. All antibodies were injected twice a week over the 4-week treatment period.

Measurement of cutaneous inflammation

Dorsal skin of the mice was injected intracutaneously with 100ng of PAF (β-Acetyl-y-O-alkyl-L-α-phosphatidylcholine from bovine heart lecithin; Sigma) or 5μg 5-HT (Sigma Aldrich) or BSA vehicle control in final volume of 50ul. After 24 and 72 hours biopsy specimens were taken for haematoxylin and eosin (H&E) staining.

PAF or serotonin receptor blockade

The PAF receptor antagonist PCA-4248 and serotonin (5-HT) receptor antagonist ketanserin tartrate were purchased from Biomol Research Labs, Inc. (Plymouth Meeting, PA). Stock solutions were prepared by dissolving PCA-4248 or ketanserin tartrate in DMSO and diluted further in PBS before injection into mice. Mice were injected with PCA-4248 i.p. (10 mg/kg) twice weekly for 4 weeks.

Measurement of skin disease severity score

A specific mouse psoriasis skin severity score composed of a rating from 0 to 3 for the symptoms of erythema, infiltration and scaling based on the macroscopic appearance of the skin was used to monitor the skin status of individual mice. Mice were evaluated in a blinded fashion once a week. Each of the symptoms was scored separately as 0 (not present), 1 (mild), 2 (moderate) or 3 (severe). The scores were summed up, taking into consideration the area involved, resulting in a maximal score of 9. In addition, skin thickness was assessed by measuring the double skin-fold thickness (DSFT) of dorsal skin of the mice with a spring-loaded engineer's micrometer (Mitutoyo Corporation) before and 48 hours after the second treatment each a week with PUVA, UVA or ethanol or antibody injection during the 4-week treatment regimen. Skin swelling was determined for individual mice by subtracting the DSFT before treatment from that after treatment at the different time points.

Tissue collection

Mice were sacrificed 48 hours after the final treatment and samples of dorsal skin, ear, spleen, lymph nodes, and blood were collected. Approximately 1 cm^2 of central dorsal skin per mouse was excised, fixed immediately in 4% buffered formaldehyde, processed routinely, and sectioned at 4 µm for haematoxylin and eosin (H&E) staining. In addition, tissue was submerged in RNAlater solution (Applied Biosystems) and stored at -70ºC for later mRNA analysis.

Assessment of microscopic skin inflammation

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 calibrated eyepiece micrometer of a microscope. Epidermal cell layers were counted at 10-15 randomly selected consecutive microscopic fields (at final magnification, x200). 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; 3, severe at 10-15 randomly selected areas per section (at final magnification, x100). The number of neutrophils in H&E sections and CD68+ cells (monocytes/macrophages) and CD3+T cells in the dermis of immunohistochemically stained sections from dorsal skin was assessed in at least 10-15 randomly selected areas per section (at final magnification, x200). 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) attached to an Olympus BX51 microscope.

Immunohistochemistry

Stainings were performed on paraffin-embedded sections of mouse dorsal skin with monoclonal anti-mouse/human TGF-β (Clone, 1D11; R&D Systems) (1:50), monoclonal anti-mouse Foxp3 (Clone, 2A11G9; Abcam) (1:100), monoclonal rat anti-mouse CD68 (clone, FA-11; Abcam) (1:50), polyclonal rabbit anti-mouse CD3 (Abcam) (1:100) or polyclonal rabbit anti-mouse IL-23p19 (Abcam) (1:500) antibody. Briefly, primary antibody was applied after pretreatment of the sections with EDTA at ph8 (for anti-IL-10, anti-TGF-β, anti-CD68, anti-CD3 and anti-IL-23p19 staining) or citric acid at ph6 (for anti-Foxp3 staining). Dako Multilink system (biotinylated polyclonal swine anti-goat, mouse, rabbit immunoglobulins, code E 0453) at a concentration of 1:50 and Dako REAL Detection System, Peroxidase/AEC, Rabbit/Mouse (code K5003) was used for antibody 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, MARM-4; Abcam) (1:100) and goat anti-rabbit IgG FITC (Abcam) (1:100), respectively, were used as secondary antibody. Cover slips were mounted onto the slides using VECTASHIELD mounting medium with DAPI (Vector Laboratories). Images were acquired by using a DP71 digital camera (Olympus) attached to an Olympus BX51 microscope.

Immunofluorescence staining

Direct or indirect stainings were performed on paraffin-embedded sections of mouse dorsal skin with phycoerythrin conjugated rat anti-mouse IL-10 (Clone, JES5-16E3; BD Pharmingen) (1:50), monoclonal anti-mouse Foxp3 (Clone, mAbcam 22510; Abcam) (1:50), goat anti-mouse IL-2Rα (CD25) (R&D System) (1:100), monoclonal rat anti-mouse IL-10 (Clone, NYRmIL-10; Abcam) (1:50) or with rabbit anti-mouse STAT3 (Abcam) (1:50). Anti-rabbit IgG FITC (Abcam) (1:100), anti-goat IgG Cy3.5 or anti-mouse IgG Cy5 were used as secondary antibody for STAT3, IL-2Rα and Foxp3 respectively. Briefly, antibody was applied after pretreatment of the sections with EDTA at pH 8 followed by blocking with 5% BSA/0.5 Tween-20. After incubation at room temperature for one hour, slides were additionally incubated with secondary antibody and/or washed and cover slips were mounted onto the slides using VECTASHIELD mounting medium with DAPI (Vector Laboratories). 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 Corporation was used to measure the levels of cytokines and chemokines in serum samples. The concentrations of the following soluble mediators were measured: G-CSF, GM-CSF, IL-1a, IL-1b, IL-4, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17, IP-10, KC, MCP-1, RANTES, TNF-α, IFN-γ, IL-2, IL-5, IL-7, IL-15, MIP-1a. Each sample was performed in duplicate according to the manufacturerer´s specifications. Standard curves for each analyte were generated by using the reference analyte concentration supplied by the manufacturers. The measurement was performed using the Bio-Plex system in combination with Bio-Plex Manager software, version 4.1, using 5-parametric curve fitting (Bio-Rad, Hercules, CA).

ELISA

IL-23 levels in serum were measured by mouse IL-23 (p19/p40) ELISA kit (eBiosciences); IL-10 levels in serum and culture supernatant were measured by mouse IL-10 ELISA kit set (BD OptEIA, BD Biosciences), according to the manufacturer's instructions. A TGF-β1-specific ELISA kit (eBiosciences) was used to quantify levels of human and mouse TGF-β1. Serum samples were acidified with 1N HCl and neutralized with 1N 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.

Multi-Analyte ELISA

Th1/Th2/Th17 cytokines Multi-Analyte ELISArray kit (SABiosciences) was used to analyze a panel of 12 cytokines involved in T helper cell biology using a conventional ELISA protocol according to the manufacturer's instructions. The cytokines represented by this array are IL2, IL4, IL5, IL6, IL10, IL12, IL13, IL17A, IL23, IFNγ, TNFα, and TGFβ1.

RNA isolation and quantitative RT-PCR

RNA was isolated from 20 mg mouse dorsal skin biopsies using QIAGEN fibrous mini kit (QIAGEN). RNA was reversely transcribed using First strand cDNA synthesis kit (Roche) and quantitative RT-PCR for cytokine transcripts and transcription factors were performed using pretested primers to IL-17A, IL17F, IL12p35, IL-12p40, IL-23p19, IL-4 and IFN-γ, RORγt, STAT3, keratin 5 (Krt5), keratin 14 (Krt14), filaggrin (Flg), and loricrin (Lor) (Super Array Biosciences Corporation). The reactions were run on an Applied Biosystems 7900HT system by using RT^2 SYBR Green / ROX qPCR Master Mix (Super Array Biosciences Corporation) for 95°C, 10 min; and then 40 cycles at 95°C, (15 sec) and 60°C (60 sec) in a total volume of 25µl. The ΔΔCt method was used to normalize transcript to GAPDH and to calculate fold-change compare to wild type control as follows: ΔΔCt = ΔCt (experimental) - ΔCt (control) (ΔCt = Ct (GOI)-Ct (HKG). Fold change = 2^(-ΔΔCt).

PAF analysis by mass spectrometry

Mouse skin was homogenized in 2 mL of phosphate bufferd saline/methanol (1/1 v/v). Each sample was spiked with 25 ng of 1-O-Octadecyl-2-butanoyl-*sn*-glycero-3-phosphocholin as internal standard immediately. Subsequently lipids were extracted according to Bligh and Dyer. Lipid extracts were taken to dryness and resuspended in 200 µL chloroform/methanol (2/1 v/v). A Thermo HyPurity Advance C18 (polar embedded), 100 x 2,1 mm, 3 µm column was used. The gradient ran from 0 to 95 % B in 1 min, then to 100% B in another 12 min where it was held for 8 min. The flow rate was 250 µL/min (4000QTrap; Applied Biosystems, coupled to a 1100 HPLC; Agilent). Data acquisition was done by MRM-scanning. The spray voltage was set to 5000 V, curtain gas to 10 psi, source temperature to 400 °C, declustering potential to 90 V and collision energy to 35 V for all analytes. The nebulizer gas was at 60 psi and the turbo gas at 50 psi. Peak areas were calculated by Analyst software for the platelet-activating factor, identified by mass, fragmentation and retention time. The amount of platelet-activating factor was calculated by standard curve method.

Cell preparation

Single cell suspension of spleen and lymph node were prepared by using Cell Dissociation Sieve (Sigma) followed by RBC lysis buffer (eBiosciences) for spleen. Single cell suspensions were prepared by mechanical disruption in RMPI 1640 (Sigma) medium supplemented with 10% (vol/vol) FCS, penicillin (100IU/ml), streptomycin (100µg/ml,) and L-glutamate (2µm; all from PAA). Prepared cells were used either for intracellular staining or for cell isolation. CASY counter was used for cell counting.

Isolation of CD4+CD25+ T regulatory cell by autoMACS Separator

CD4+CD25+ T cells were isolated by mouse Treg cell isolation kit (Militini Biotech) from spleen single cell suspension. For the isolation of CD4+ T cells, non-CD4+ T cells are indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies (CD8a (Ly-2), CD11b (Mac-1), (CD45R (B220), CD49b (DX5), and Ter-119) and then Anti-Biotin MicroBeads (conjugated with monoclonal anti-biotin antibody). In parallel, the cells are labeled with CD25-PE (clone: 7D4). The cell suspension is loaded onto a MACS column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled non-CD4+ T cells are retained in the column, while the unlabeled CD4+ T cells run through. For the isolation of CD4+CD25+ T cells, the CD25+ PE-labeled cells in the enriched CD4+ T cells fraction are magnetically labeled with Anti-PE MicroBeads. The cell suspension is again loaded onto a column which is placed in the magnetic field of a MACS Separator. The magnetically labeled CD4+CD25+ cells are retained in the column, while the unlabeled cells run through. After removal of the column from the magnetic field, the retained fraction CD4+CD25+ T cells are eluted as the positively selected cell fraction. Isolated cells were more than 95% pure.

Regulatory T cell suppression assay

To analyze proliferation and suppressive capacity of CD4+CD25+ Tregs in response to polyclonal activation, CD4+CD25+ or CD4+CD25- T cells (1×10^5) were cultured in RPMI 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 ^{3}H-thymidine (Amersham Pharmacia Biotech) per well added for the final 16 h of the 72 h incubation. Cell harvesting performed by using Perkin Elmer FilterMate cell harvester on 96-well plate and after harvesting beta counting was done on Perkin Elmer MicroBeta by using scintillation cocktail.

Functional analysis of regulatory T cells

Proliferation assay of CD4+CD25+ T regulatory cells, CD4+CD25- T responder cells or their co-culture were performed from PUVA 0.25 J/cm^2 treated mice. Each cell subset was cultured in duplicate either alone (1 x 10^5) or with an equal number (1:1) of CD4+CD25+ and CD4+CD25- cells, in the absence or presence of 2.5ug, 5ug or 10ug concentration of anti-CTLA4 monoclonal antibody 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 ^{3}H-thymidine (Amersham Pharmacia Biotech) per well added for the final 16 h of the 72 h incubation. Cell harvesting performed by using Perkin Elmer FilterMate cell harvester on 96-well plate and after harvesting beta counting done on Perkin Elmer MicroBeta by using scintillation cocktail.

Intracellular IL-10 and Foxp3 staining

Isolated CD4+CD25+ T cells (1x10^5) were cultured in triplicates, as described above. Cells were first stained for CD4 surface antigen and then fixed in eBiosciences 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 eBiosciences permeabilization buffer and stained for Foxp3 according to the manufacture's instructions.

Phenotypic analysis of Foxp3+ regulatory T cells

Lymph node or spleen cells were collected after PUVA treatment for Foxp3+ regulatory T cell phenotype (CD4+CD25+Foxp3+) by using mouse Treg staining kit (eBiosciences). Cells were first stained with surface marker CD4 (0.125μg/test) and CD25 (0.06μg/test) one by one in a final volume of 100μl flow staining buffer. After fixation/permeabilization cells were further stained with Foxp3 (0.5μg; clone, FJK-16s) according to the manufacture's instructions.

Intracellular staining of IL-17A, IL-17F and IFN-γ

Intracellular cytokine staining was performed on spleen single cell suspension or isolated CD4+ T cells. Cells ($1x10^5$) 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 RMPI 1640 medium supplemented with 10% (vol/vol) FCS, penicillin (100IU/ml), streptomycin (100 µg/ml) and L-glutamate (2µm) in triplicates for 4 days then leukocyte activating cocktail (BD Pharmingen) added for 5 hours in round-bottom 96-well plate (Nunc). After washing cells were first stained for CD4 surface antigen and then treated with Fix/Per buffer (eBiosciences) according to the manufacturer's instructions. Intracellular cytokine staining was performed using antibodies to IFN-γ, IL-17A and IL-17F.

Analysis of Homing receptor

Analysis of homing receptors on CD4+ T or CD4+CD25+ regulatory T cells were performed on single cell suspension of lymph node. CCR7, CD62L, MHC-II and CLA were used as homing markers. Cells were first stained with surface antigen CD4 in flow cytometry staining buffer and then after washing the cells in flow cytometry staining buffer, cells were further stained with homing markers. For homing of CLA+CD25+CD4+ regulatory T cells; cells were first stained with CD4 and CD25 and then after washing further stained with purified primary antibody of CLA and fallowed by staining with goat anti-rat IgG secondary antibody (Invitrogen GmbH) after washing. Plots are either gated on total lymphocytes or CD4+ T cells. FACS data were acquired on a FACSCalibur flow cytometer and data were analyzed with CellQuest Pro software (BD Biosciences). Numbers in quadrants indicate percent cells. Data shown are from one representative experiment, with n=3 pooled mice per treatment group.

FACS analysis

FACS data were acquired on a FACSCalibur flow cytometer and data were analyzed with CellQuest Pro software (BD Biosciences). All plots were gated on CD4+ T cells or CD4+C25+ T regulatory cells.

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 analysis of variance (ANOVA), 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 P-level < 0.05.

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11. ABBREVIATIONS

Abbreviation	**Definition**
APC	Antigen presenting cell
CCL	Chemokine (C-C motif) ligand
CXCL	Chemokine (C-X-C motif) ligand
CCR	C-C chemokine receptor
CXCR	CXC chemokine receptor
CD	Cluster of differentiation
CTLA4	Cytotoxic T-lymphocyte antigen 4
CD62L	L-selectin, adhesion molecule
COX2	Cyclooxygenase 2
CLA	Cutaneous leucocyte-associated antigen
DCs	Dendritic cells
DNA	Deoxy ribonuclic acid
DSFT	Double skin folds thickness
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
FOXP3	Forkhead box P3
Flg	Filaggrin
G-csf	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GITR	Glucocorticoid-induced TNFR-related protein
iNOS	Inducible nitric oxide synthase
KGF	Keratinocyte growth factor
Krt5	Keratin 5
Krt14	Keratin 14
KC	Keratinocyte-derived chemokine
IL	Interleukin
iTrge	Induced regulatory T cell
Ig	Immunoglobin
LCE3B	Late cornified envelope 3B
Lor	Loricrin
IFN	Interferon
ICAM	Inter-cellular adhesion molecule 1

MHC	Major histocompatibility complex
MIP	Macrophage inflammatory proteins
MPD	Minimal phototoxic dose
MCP	Monocyte chemoattractant protein
NK cells	Natural Killer cells
PAF	Platelet activating factor
PLA2	Phospholipase A 2
PSGL-1	P-selectin glycoprotein ligand-1
POSORS	Psoriasis susceptibility gene
PGE2	Prostaglandin E2
PUVA	Psoralen plus ultra violet A
ROR-γT	RAR-related orphan receptor gammaT
STAT3	Signal transducer and activator of transcription 3
TARC	Thymus- and activation-regulated chemokine
Th	T helper cell
TNF	Tumor necrosis factors
TNFAIP3	Tumor necrosis factor alpha-induced protein 3
TGF	Transforming growth factor
Tc1	Cytotoxic T cell type 1
TCR	T cell receptor
Tregs	Regulatory T cells
UVB	Ultraviolet B
VCAM	Vascular cell adhesion protein
WT	Wild type
ZNF313	Ginc finger protein gene 313

Declaration

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Modulation of Th1/Th2 immunity and airway inflammation by the drug MIL004 in mouse model of asthma.

Technical expertise/experiences:

Development of disease mouse models, FACS analysis, T cell isolation and characterization, proliferation and suppression assays, ELISA, EMSA, western blot, qRT-PCR, immunohistochemistry, in vitro and in vivo siRNA gene silencing, FLEX assay, Luciferace reporter gene assay, NFAT translocation assay, Ca2+ imaging, cell culture, evaluation of disease phenotype, gene sequencing, genotyping and tumour biology and radiation related techniques (UVA and UVB). Worked on TGF-β1 transgenic, Cblb -/- and Jun ko mice.

Awards:

2010 Travel Award by 40th annual ESDR meeting for the oral presentation at Helsinki, Finland.

2009 Best Poster Award at joint ZMF-Doctoral day, Medical University of Graz, Graz, Austria.

2007 Abstract was selected for travel Award by 4th Indian Australian Conference on Biotechnology for the oral presentation at the Queensland Institute of Medical Research, Brisbane Australia (Co-author).

2006 Best Poster Award by XXXI Annual conference of Indian society of Human Genetics, Delhi, India (Co-author)

2004 Awarded certificate of appreciation by the Ambedkar Center For Biomedical Research (ACBR), New Delhi in National Conference on Emerging Areas in Biomedical Sciences.

Memberships in professional societies:

2009-current European Society for Dermatological Research (ESDR)

Publications:

Full paper/article (Journal)

Singh TP, Schon MP, Michaelis K, Wallbrecht K, Rinner B, Mayer G, Schmidbauer U, Strohmaier H, Wang XJ, Wolf P
8-methoxypsoralen plus UVA therapy acts via inhibition of the IL-23/Th17 axis and induction of Foxp3+ Tregs involving CTLA4 signaling in a psoriasis-like skin disorder.
The Journal of Immunology 2010 Jun 15;184(12):7257-67

Singh TP, Huettner B, Koefeler H, Wallbrecht K, Schon MP, Bambach I, Wolf P
Platelet-activating factor blockade inhibits Th17 pathway and suppresses psoriasis-like skin disorder in K5.hTGF-β1 transgenic mice.
The American Journal of Pathology (Submitted, in revision)

Wackernagel AG, Heinemann A, Konya V, Byrne SN, **Singh TP**, Legat F, Hofer A, Wolf P
Photohardening restores the impaired neutrophil responsiveness to chemoattractants in patients with polymorphic light eruption
Experimental Dermatology (submitted)

Batra J, **Pratap Singh T**, Mabalirajan U, Sinha A, Prasad R, Ghosh B.
Association of inducible nitric oxide synthase with asthma severity, total serum immunoglobulin E and blood eosinophil levels.
Thorax 2007Jan; 62(1):16-22.

Sharma S, Mann D, **Singh TP**, Ghosh B.
Lack of association of histamine N-methyltransferase (HNMT) polymorphisms with asthma in the Indian population.
Journal of Human Genetics 2005; 50(12):611-617.

Abstract (Journal)

Singh TP, Huettner B, Koefeler H, Mayer G, Wallbrecht K, Schon MP, Wolf P
Platelet-activating factor blockade inhibits Th17 pathway and suppresses psoriatic-like skin disease in K5.hTGF-beta1 transgenic mice. 40th ESDR meeting, Sep 07-12, 2010; **Helsinki, Finland**.
Abstract was selected for travel award [Oral Communication]
Journal of Investigative Dermatology. 2010; 130: S3-S3.

Heinemann A, Wackernagel AG, Konya V, Byrne SN, **Singh TP**, Legat F, Hofer A, Wolf P
Photohardening restores the impaired neutrophil responsiveness to chemoattractants in patients with polymorphic light eruption. 40th ESDR meeting, Sep 07-12, 2010; **Helsinki, Finland**. **[Poster]**
Journal of Investigative Dermatology. 2010; 130: S48-S48.

Singh TP, Schon MP, Michaelis K, Wang XJ, Wolf P
Treg/Th17 axis in 8-methoxypsoralen plus ultraviolet-A response of a psoriasis-like skin disorder in mice
39th ESDR meeting, Sep 09-12, 2009; **Budapest, Hungary**. **[Oral Communication]**
Journal of Investigative Dermatology. 2009; 129: S32-S32.

Singh TP, Schon MP, Michaelis K, Wang XJ, Kerl H, Wolf P
Th17/Treg paradigm: effect of 8-methoxypsoralen plus ultraviolet A in a mouse model of psoriasis
5th International Congress on Psoriasis - From Gene to Clinic; Dec 04-06, 2008; **London, England**.
Travel was supported by Wyelth Lederle Pharma GmbH, Austria. [Oral Communication]
British Journal of Dermatology. 2008; 159(6):1398-1398.

Singh TP, Schon MP, Michaelis K, Wang XJ, Kerl H, Wolf P
Psoralen plus UVA lowers IL-23 levels and clears psoriasis-like skin lesions in TGF-beta1 transgenic mice**.**
34th International congress on Dermatology, **Kyoto, Japan [Poster]**
Journal of Investigative Dermatology. 2008; 128: S201-S201.

Abstract (Proceedings)

Singh TP, Schon MP, Michaelis K, Wallbrech K, Rinner B, Mayer G, Schmidbauer U, Strohmaier H, Wang XJ, Wolf P
Induction of IL-10 producing CD4+CD25+Foxp3+ T regulatory cells and inhibition of the inflammatory IL-23/Th17 pathway are central for the therapeutic effect of psoralen+UVA photochemotherapy.
Joint ZMF-Doctoral day 2009, **Graz, Austria**. **Presented work was selected for best poster award. [Poster]**

Singh TP, Huettner B, Koefeler H, Wolf P
An important role of platelet activating factor (PAF) and its receptor in pathogenesis of psoriasis-like skin inflammation. Joint ZMF-Doctoral day 2009, **Graz, Austria. [Poster]**

Wolf P, **Singh TP**
Advances in photomedicine: Better understanding of molecular mechanisms of photo(chemo)therapy and novel combination treatment strategies. 13th Congress of the European Society for Photobiology; September 5-10; **Wroclaw**. 2009. **[Oral Communication]**

Singh TP, Schon MP, Michaelis K, Wang XJ, Kerl H, Wolf P
TGF-beta1 transgenic mice: a suitable model for the therapeutic action of PUVA in psoriasis **.**
34th Meeting of the American Society for Photobiology; June 20-25, 2008; **Burlingame, CA, USA**. 2008. **[Poster]**

Batra J, **Singh TP**, Mabalirajan U, Sinha A, Prasad R, Ghosh B.
Association of inducible nitric oxide synthase (inos) with asthma severity, total serum IgE and blood eosinophil counts. The Australian Health & Medical research Congress, **Brisbane, Australia,** 16-21 November 2008. **Abstract was selected for oral presentation.**

Singh TP, Ram A.
Modulation of Th1/Th2 immunity and airway inflammation by the drug MIL004 in mouse model of asthma. National Conference on Tribal Health, organized by Regional Medical Research Center for Tribes, **Jabalpur M.P, India**, November 2007. **[Poster]**

Batra J, **Singh TP**, Mabalirajan U, Sinha A, Prasad R, Ghosh B.
Functional microsatellite repeats in Inducible Nitric Oxide Synthase (iNOS) are associated with asthma severity, serum IgE levels and blood eosinophil counts. 4th Indo Australian Conference on Biotechnology, 7-9 May 2007, **Brisbane, Queensland, Australia**. **Abstract was selected for the oral presentation and for the Travel Award.**

Batra J, **Singh TP**, Mabalirajan U, Sinha A, Prasad R, Ghosh B.
Association of inducible nitric oxide synthase (inos) with asthma severity, total serum IgE and blood eosinophil counts. 33rd Indian Immunological Society Conference**, Delhi, India**, 28th -31st January 2007. **Presented work was selected for the best presentation award.**

Batra J, **Singh TP**, Mabalirajan U, Sinha A, Prasad R, Ghosh B.
Association of inducible nitric oxide synthase (iNOS) with asthma. International Symposium on Human Genomics and Public Health and XXXI Annual conference of Indian society of Human genetics, **Delhi, India**; February 2006. **Presented work was selected for the best poster award.**

Chatterjee R, **Singh TP,** Ghosh B.
Acidic Mammalian Chitinase (AMCase) as a candidate gene for Atopic Asthma**.**
International Symposium on Human Genomics and Public Health and XXXI Annual conference of Indian society of Human genetics, **Delhi India**, February 2006.

Ram A, Mabalirajan U**, Singh TP**, Muthukumar S.P, Rengarajan S, Ghosh B.
Attenuation of Airway Hyper responsiveness and Pulmonary Inflammation by a Natural Compound, MIL 0004 in Mouse Model of Asthma. 2nd national conference on BioTech 2004 Challenges & Opportunities, **Delhi, India**.

Singh TP. Gama-amino butyric acid drugs (GABA Drugs).
National Conference on Emerging Areas in Biomedical Sciences, organized by Ambedkar Center For Biomedical Research (ACBR), New Delhi and Bundelkhand University, **Jhansi, India**, December 2004. **[Poster]**

Acknowledgements in scientific papers:

Association of a chromosome 1q21 locus in close proximity to a late cornified envelope-like proline-rich 1 (LELP1) gene with total serum IgE levels.
J Hum Genet. 2007;52(4):378-83.

Genetic landscape of the people of India: a canvas for disease gene exploration
India Genome Variation Consortium 2008.
J. Genet 2008 Apr;87(1):3-20.