Dissertation

Pathomechanisms of polymorphic light eruption

submitted by

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ABBREVIATIONS

CD	Cluster of differentiation
CHS	Contact hypersensitivity
CXCR4	C-X-C motive-chemokine receptor 4
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
EM	Erythema multiforme
GITR	Glucocorticoid-induced tumor necrosis factor receptor
G/L	Giga per Liter
GN	Glomerulonephritis
HSP	Heat shock protein
HSV	Herpes simplex virus
25(OH)D	25-hydroxyvitamin D_3
ICAM	Intercellular Adhesion Molecule 1
IgE	Immunoglobulin E
IL	Interleukin
LCs	Langerhans cells
PLE	Polymorphic light eruption
PUVA	Psoralen + UVA
RANKL	Receptor activator of NF-κB ligand
TGF-β	Transforming growth factor- β
Th1-cells	T helper 1 cells
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
UV	Ultraviolet

ABSTRACT

The exact pathomechanisms of polymorphic light eruption (PLE) are still unknown, but recent evidence suggests a decreased infiltration of neutrophils, macrophages and mast cells upon UV radiation into the skin, which is associated with non-emigration of Langerhans cells (LCs) that are important for the immune response. In contrast, in irradiated skin of healthy individuals LCs migrate to the draining lymph nodes where they might activate regulatory T cells (Treg) which suppress cellular immunity by suppression of auto-reactive T cells. The skin lesions of patients are abating as summer progresses due to a so called "hardening effect" which can also be induced by prophylactic administration of medical photo(chemo) therapy like PUVA, 311nm UVB or UVB.

In the present work possible effectors were investigated, that if missing or dysfunctional may render patients with photodermatoses more susceptible to UV radiation. In study part 1 Treg numbers and function were assessed in 30 PLE patients with and without medical phototherapy as well as 19 healthy controls at two time points from March to June of a season from 2012 to 2014. Medical photohardening of 23 PLE patients resulted in a significant increase in the median percentage of circulating Treg (65,6% increase in lymphocyte population (P=0.0049), 32,5% increase in $CD4^+$ T cell population; (P=0.0049)). This was accompanied by an increase in the expression of *FoxP3* mRNA (P=0.0083) and relative immunosuppressive function of Treg (P=0.084), comparing the two time points in representative subsets of patients with healthy controls tested. A tendency towards an increase in Treg numbers of PLE patients without medical photohardening was observed, suggesting a natural photohardening by seasonal factors. An increase in Treg levels might counteract the susceptibility to PLE. The latter finding is substantiated by the results of the second part of the thesis where the effects of season on vitamin D levels as well as Treg numbers and function in PLE patients were investigated. A positive correlation of season with vitamin D serum levels (Spearman R = 0,586, Spearman P = 0,0026) was observed. There was no relationship of season or Vitamin D with Treg levels and function, although a seasonal tendency for an increase in relative and absolute Treg numbers was detected. Together this indicates that Treg numbers and Treg suppressive function in PLE patients is independent from 25(OH)D serum levels. By using the mast cell-deficient $Kit^{W\text{-}Sh/W\text{-}Sh}$ mouse model in thesis part 3 a potential mechanistic role of mast cells in reducing the sensitivity to UV radiation through photohardening was determined, identifying a new *in vivo* model to study photodermatoses. The results from this ani-

mal model suggested that mast cells initiated phototolerance through photohardening and provided a recruiting signal to neutrophils and Treg upon UV radiation. While it is well known that mast cell-deficient mice are resistant to UV-induced immune suppression, it was discovered that they are prone to develop photo itch and are more susceptible to UV-induced epidermal hyperplasia as well as skin edema.

Together these results indicate that Treg are involved in the pathophysiology of PLE. In addition it is proposed that Treg are affected by UV radiation and/or other potential seasonal factors.

ZUSAMMENFASSUNG

Die genauen Pathomechanismen der polymorphen Lichtdermatose (PLD) sind noch immer unbekannt, aber jüngste Studienergebnisse deuten auf eine verminderte Infiltration von neutrophilen Granulozyten, Makrophagen und Mastzellen in die UV-bestrahlte Haut hin, was zu einem Verbleib der für die Immunantwort wichtigen Langerhans Zellen (LZ) in der Haut führt. Im Gegensatz dazu wandern die LZ in UV-bestrahlter Haut gesunder Individuen zu den Lymphknoten, wo sie möglicherweise regulatorische T-Zellen (Treg) aktivieren, welche eine zelluläre Immunantwort hervorgerufen durch autoreaktive T-Zellen supprimieren. Die Hautsymptomatik der Patienten schwächt sich gewöhnlich im Sommer in Folge weiterer Sonneneinwirkung durch einen sogenannten „hardening-Effekt“ ab, welcher auch durch eine medizinische Photo(chemo)therapie wie PUVA, 311nm UVB oder UVB induziert werden kann.

In der vorliegenden Arbeit wurden mögliche Effektoren untersucht welche, wenn fehlend oder dysfunktional, Patienten mit Photodermatosen empfänglicher für UV-Bestrahlung machen könnten. Für den ersten Teil der Dissertation wurden die Anzahl der Treg und deren Funktion in 30 PLD-Patienten mit und ohne medizinisches Photohardening sowie jene von 19 gesunden Probanden an zwei Zeitpunkten von März bis Juni einer Saison von 2012 bis 2014 untersucht. Medizinisches Photohardening von 23 PLE-Patienten führte zu einem signifikanten Anstieg an zirkulierenden Treg (mit einem Medianwert von 65,6% in der Lymphozytensubpopulation ($P = 0.0049$) und 32,5% in der CD4+-Subpopulation ($P = 0.0049$)). Dies wurde von einer Zunahme der *FoxP3* mRNA Expression ($P = 0.0083$) sowie der relativen immunsuppressiven Funktion der Treg ($P = 0.084$) durch einen Vergleich der zwei Zeitpunkte bei einer repräsentativen Anzahl an Patienten und gesunder Kontrollen begleitet. Eine Tendenz für einen Anstieg der Treg-Anzahl bei PLD-Patienten ohne medizinisches Photohardening konnte beobachtet werden, welches auf ein natürliches Photohardening durch saisonale Faktoren hinweist. Eine Erhöhung der Treg-Anzahl könnte einer Anfälligkeit gegenüber PLD entgegenwirken. Letztere Beobachtung wurde durch die Ergebnisse des zweiten Teils der Dissertation gestützt, bei dem der saisonale Einfluss auf die Vitamin-D-Serumspiegel sowie auf die Anzahl der Treg und deren Funktion bei PLD-Patienten untersucht wurde. Eine positive Korrelation der Saison mit den Vitamin-D-Serumspiegeln (Spearman $R = 0{,}586$; Spearman $P = 0{,}0026$) wurde beobachtet. Es gab keinen Zusammenhang der Saison oder der Vitamin-D-Spiegel mit der Treg-Anzahl und deren Funktion, obwohl eine saisonale Tendenz für einen Anstieg der relativen und absoluten Treg-Anzahl beobachtet wurde. Zusammen betrachtet

weisen diese Ergebnisse daraufhin, dass die Treg-Anzahl und deren suppressive Funktion bei PLD-Patienten unabhängig von 25(OH)D-Serumspiegeln sind. Durch Verwendung des mastzelldefizienten Mausmodells $Kit^{W\text{-}Sh/W\text{-}Sh}$ im dritten Teil der Dissertation wurde die mechanistische Rolle der Mastzellen bei der Verringerung der Empfänglichkeit gegenüber UV-Strahlung durch Photohardening bestimmt, wobei hiermit ein neues *in-vivo*-Model für die Untersuchung von Photodermatosen identifiziert wurde. Die Ergebnisse wiesen darauf hin, dass Mastzellen eine Phototoleranz durch Photohardening initiierten und ein Rekrutierungssignal für neutrophile Granulozyten und Treg nach einer UV-Bestrahlung bereitstellten. Während bekannt ist, dass mastzelldefiziente Mäuse resistent gegenüber einer UV-induzierten Immunsuppression sind, wurde entdeckt, dass diese anfälliger sind für UV-induzierten Juckreiz und empfänglicher für UV-induzierte epidermale Hyperplasie sowie Ödem der Haut.

Zusammengefasst weisen diese Ergebnisse daraufhin, dass Treg in die Pathophysiologie der PLD involviert sind. Des Weiteren wird vorgeschlagen, dass Treg durch die UV-Strahlung und/oder andere potentielle saisonale Faktoren beeinträchtigt werden.

1. INTRODUCTION

1.1 Polymorphic light eruption (PLE)

Polymorphic light eruption ("sun allergy") is the most common idiopathic photosensitivity disorder, affecting 10 to 20% of young woman in the first three decades of life (Hönigsmann, 2008; Pao et al., 1994; Stratigos et al., 2002). The disease is characterized via itchy lesions of different morphology that usually appear on sun-exposed body sites after first sun exposure in spring or early summer (Stratigos et al., 2002; Tutrone et al., 2003). The rash is known to appear within hours to days after exposure, but subsides within several days without scarring (Jansen, 1979; Van Praag et al., 1994). Its incidence increases towards higher northern latitudes and fair-skinned individuals (Fitzpatrick classification I-IV) are more likely to be affected then other skin types. This accumulative appearance in temperate regions may be due to the high differences in UV radiation between summer and winter and a consequential loss of photoadaption of the skin (Morison and Stern, 1982; Pao et al., 1994; Ros and Wennersten, 1986; Tutrone et al., 2003). A so called "skin hardening effect" arises as summer progresses, as people are becoming gradually less sensitive to sunlight and episodes are less likely to appear or are less severe than in spring. This phenomenon leads to a complete tolerance even of high sun dosages towards the end of summer, but there is a likely recurring of PLE on an annual basis each spring or early summer (Jansen, 1979; Naleway, 2002). Medical phototherapy or photochemotherapy (broadband UVA, PUVA, broadband UVB or narrowband 311-nm UVB) is effective in most PLE patients, since it simulates a hardening effect and aims to induce photoadaption with regulated doses of UV radiation (Naleway, 2002; Wolf et al., 2009). The exact mechanism remains unknown, but thickening of the stratum corneum, skin melanization and/or an immunological mechanism are thought to play a role upon repetitive UV radiation (Ferguson and Ibbotson, 1999; Norris and Hawk, 1990; Wolf and Oumeish, 1998).

1.2 UV radiation and photo-antigens

The action spectrum of triggering PLE spans the UVB (280nm – 320nm) and UVA (320nm – 400nm) region (Hölzle et al., 1982; Lindmaier and Neumann, 1991; Mastalier et al., 1999), whereas Boonstra et al. (Boonstra et al., 2000) discovered that in some cases even visible light (400nm – 780nm) was found to provoke lesions in patients (Figure 1). In this photoprovocation studies PLE patients were generally more susceptible to UVA, a finding that is substantiated by the increased prevalence of PLE in temperate regions which have a greater UVA proportion in spring and autumn compared to UVB (Pao et al., 1994). In summer the proportion reverses and may inhibit episodes in patients through an UVB-induced immunosuppressive mechanism (Baadsgaard et al., 1987).

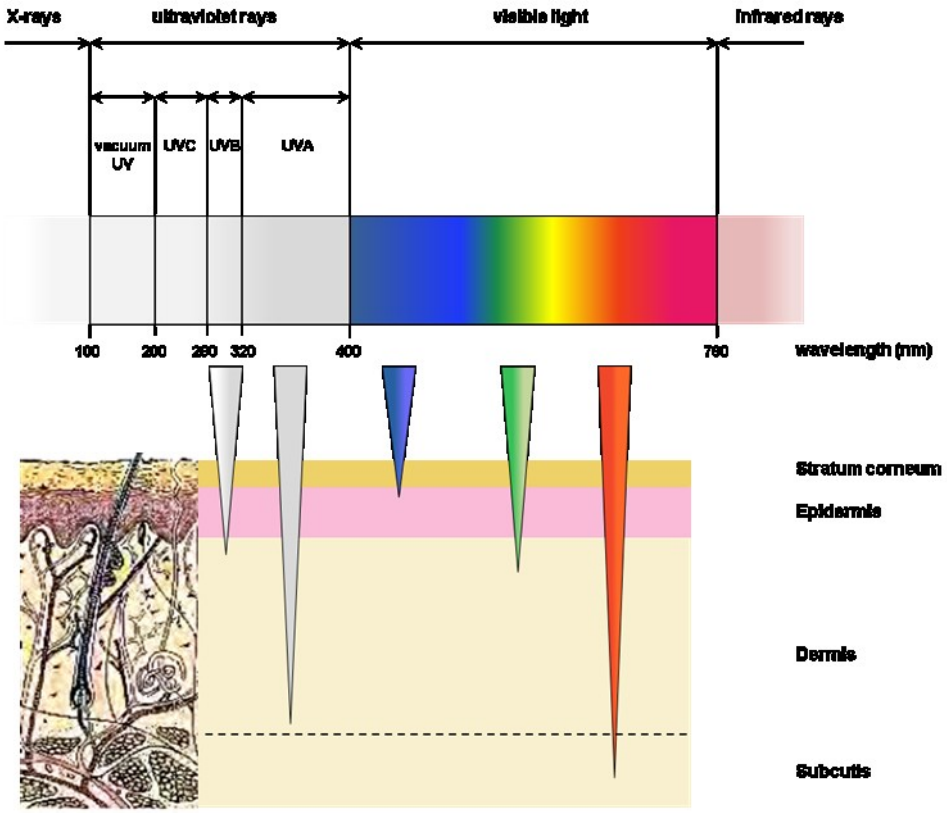

Figure 1: *Electromagnetic spectrum of radiation and penetration of the skin. The UV light is part of the electromagnetic spectrum, located between X-rays and visible light. The depth of penetration through the skin is dependent on the wavelength. UVC is nearly completely blocked by the ozon layer. UVB does not penetrate the skin as deeply as UVA or red light, but is more energetic. Very short (gamma and cosmic-rays) and long (microwave and radio-rays)*

wavelengths are not shown. Adapted from (Gupta et al., 2013) *and (http://www.waldmann.com/waldmann-medizin/home/home/topics/light_sources_for_phototherapy.en.html).*

This characteristic of UV radiation can also inhibit the host anti-tumor response as shown by a study of Fisher and Kripke, who demonstrated that immunogenic skin tumors which normally would be rejected by the host's immune system, do grow upon chronic UV radiation of the recipient mice prior to tumor inoculation (Fisher and Kripke, 1977).

The UVB radiation is also known to effectively activate photochemical reactions and modify lipids, DNA or proteins (de Gruijl, 1997; Pattison and Davies, 2006; Ravanat et al., 2001). These newly formed antigens have the potential to elicit an autoimmune response, while at the same time the immunosuppressive property of UV radiation may prevent this adverse reaction in healthy subjects (Cooper, 1996; Cooper et al., 1992; Yoshikawa et al., 1990). Heat shock protein 65 (HSP65) might represent a likely photo-induced antigen playing a role in the appearance of PLE, since its expression was increased in endothelial cells and epidermal keratinocytes in experimentally induced PLE lesions and persisted further on in dermal dendritic cells for 6 days (McFadden et al., 1994). This was in contrast to healthy controls, where an increase was not detected. Another possible photoantigen theoretically triggering PLE might be Herpes simplex virus (HSV), which can cause erythema multiforme (EM), a recurring (muco)cutaneous disorder, sharing similarities to PLE (Fraser-Andrews et al., 2005; Wolf et al., 1994). PLE patients taking the anti-viral substance acyclovir stated to be free of PLE symptoms (Baby, 2002), whereupon Wackernagel et al. (Wackernagel et al., 2006) investigated skin samples of PLE patients by polymerase chain reaction and southern blot analysis for HSV DNA. However, they did not detect any presence of HSV DNA in the samples.

1.3 UV-induced immune suppression

The pathogenesis of PLE remains unknown, but a resistance to UV-induced immune suppression resulting in an immune response directed against UV-modified skin seems to play a role. In healthy individuals interleukin (IL)-4 and IL-10 are secreted together with Prostaglandin E_2 upon UV radiation to give rise to UV-induced immunosuppression (Shreedhar et al., 1998). This subsequently leads to the infiltration of HLA-DR1/CD11b1/CD1a- macrophages into and a migration of Langerhans cells (LCs) out of the skin to the draining lymph nodes, where they

play a key role in inducing immunologic tolerance (Noonan et al., 1984; Toews et al., 1980; Ullrich, 1995).

This is in contrast to PLE patients where the skin is infiltrated by very few macrophages upon irradiation with high dosages of UVB which additionally comprise of CD68-positive cells, whereas the ones of healthy controls were found to be CD68-negative (Kölgen et al., 1999). The same group also showed that contrary to healthy individuals CD1a+ epidermal LCs are residing in the skin of PLE patients upon UVB radiation (Kölgen et al., 1999), a finding that is supported by Norris et al. who observed increased levels of LCs in skin biopsies of patients 1h and 5h post-irradiation (Norris et al., 1989). Contradictory to these observations Wackernagel et al. found no differences in the disposition of LCs between PLE patients and healthy volunteers (Wackernagel et al., 2004). Possible explanations for this different outcome can be found in the diverse setup of the studies, including UV spectra, doses and sampling.

UVB-exposed skin is also infiltrated by a large number of IL-4-positive neutrophils that via IL-10 secretion favor Th2-cell induction (Teunissen et al., 2002). IL-10 is a very potent immunosuppressive cytokine and its blocking in UVB–exposed mice resulted in inhibition of UVB-induced immunosuppression (Beissert and Granstein, 1996) and photocarcinogenesis (Loser et al., 2007). In addition to IL-10, IL-4 also plays a major role in UVB-induced immunosuppression, since delayed type hypersensitivity (DTH) reactions are not inhibited upon UVB exposure in IL-4 knockout mice infected with HSV (el-Ghorr and Norval, 1997). It was demonstrated that the migration of neutrophils into the skin of PLE patients was impaired after UVB irradiation, compared to healthy subjects (Schornagel et al., 2004). This may subsequently lead to a decreased local production of IL-10 and IL-4. The cytokine milieu in the skin might be altered, resulting in a diminished immune suppression and Th1-cell skewing. This is supported by the finding of reduced expressions of TNF-alpha, IL-4 and to a lesser extend IL-10 in UVB-irradiated PLE skin (Kölgen et al., 2004), which may favor the development of a Th-1 response, though the group could not detect any differences in Th-1 skewing cytokines between healthy controls and patients.

Schornagel et al. investigated the infiltration mechanism by analyzing the expression of the adhesion molecules ICAM-1 and E-selectin in PLE patients and healthy controls (Schornagel et al., 2004). They did not detect any differences between both groups and observed a similar

increase of the proteins 6h after irradiation which was also reflected on the chemotactic responses of neutrophils to IL-8 and CD5a. PLE patients were shown to display a dysfunctional neutrophil responsiveness to leukotriene B4 and formylmethionylleucyl-phenylalanine that is restored after medical photohardening (Gruber-Wackernagel et al., 2011). The photohardening therapy also re-established the LCs migration in PLE patients (Janssens et al., 2005), but it is still not clear, whether a local and/or systemic mechanism leads to the impaired neutrophil infiltration.

1.4 Vitamin D

Another factor that might theoretically play a role in the pathogenesis of PLE is vitamin D. It has been demonstrated that latitude, UV exposure, autoimmune diseases mediated by Th-1 and vitamin D insufficiency might be connected, since autoimmune disease incidence correlated positively with increasing latitude (Cantorna, 2000; Ponsonby et al., 2002). Recent evidence suggests a significance of vitamin D in regulating a plethora of Th1-mediated autoimmune disorders, like SLE (Kamen and Aranow, 2008), diabetes mellitus type I (Mathieu et al., 1994), rheumatoid arthritis (Aguado et al., 2000) and inflammatory bowel disease (Froicu et al., 2003). Patients with systemic lupus erythematosus (LE), multiple sclerosis and rheumatoid arthritis exhibit low vitamin D serum levels that in case of LE correlate with disease severity (Cutolo and Otsa, 2008). In multiple sclerosis patients high serum levels of vitamin D positively correlated with the suppressive function of $CD4^{+}CD25^{+}CD127^{-}$ Tregs, proposing it as an important mediator of T cell regulation via inhibition of Th1 and Th17 cells (Correale et al., 2009; Smolders et al., 2009, 2008).

Since a similar pathogenesis for LE and PLE has been suggested (Millard et al., 2001b, 2001c), a study was conducted where PLE patients pretreated their skin with the 1,25-dihydroxyvitamin D_3 analogue containing calcipotriol-or placebo cream before starting photoprovocation testing (Gruber-Wackernagel et al., 2011). The calcipotriol-containing cream reduced PLE symptoms in all patients tested. It can be hypothesized that the mechanism by which photohardening and vitamin D ameliorate PLE might involve Tregs. Indeed, vitamin D supplementation was associated with significantly increased numbers of Tregs within circulating CD4+ cells in apparently healthy individuals (Bock et al., 2011; Prietl et al., 2010). In addition, vitamin D3 enabled by IL-2 modulated the regulatory activity of CD4+CD25+ T cells (Gorman et al., 2010).

1.5 Regulatory T cells (Tregs)

Tregs mature in the thymus and represent a subpopulation of T cells that mediate immunologic self-tolerance (Sakaguchi, 2001). They express a plethora of intracellular and surface markers including CD4, CD25 (Fehérvari and Sakaguchi, 2004), FoxP3 (Fontenot et al., 2003; Hori et al., 2003), GITR (McHugh et al., 2002), CD152 (CTLA-4) (Sansom and Walker, 2006), neuropilin-1 (Sarris et al., 2008) and Helios (Akimova et al., 2011). If absent or dysfunctional, severe multiorgan autoimmunity develops in humans and mice (d'Hennezel et al., 2012; Sakaguchi et al., 2010). Tregs and their function are thought to play a crucial protective role in various inflammatory skin diseases like atopic dermatitis (Ou et al., 2004) and lupus erythematosus (Valencia et al., 2007). Sugiyama et al. demonstrated that Tregs from psoriatic skin lesions had an impaired suppressive function on the proliferation of effector T cells, which may subsequently lead to hyperproliferation of pathogenic T cells in psoriasis (Sugiyama et al., 2005).

Concerning PLE, where the normal immune suppressive response to UV radiation is defective, it was hypothesized that PLE patients might display a fluctuating deficiency in their Treg levels and/or their suppressive function (Wolf et al., 2009), which may be linked to seasons where Treg levels are low and their suppressive capacity is dysfunctional. This has now been supported by a recent observation by Gambichler et al. showing an impaired Treg infiltration together with a decreased expression of IL-10, TGF-β1, and RANKL in UVA1-exposed skin lesions of PLE patients (Gambichler et al., 2013).

It was shown that a single, low solar-simulated irradiation that was given prior to the elicitation phase of contact hypersensitivity (CHS), led to photoimmune suppression mediated by IL-10 producing Tregs (Stoebner et al., 2006). Medical photohardening may restore Treg levels or function by inducing IL-10 producing Tregs in PLE patients, which were shown to lack IL-10 upon UV irradiation (Kölgen et al., 2004).

The mast cell might represent another key player that may contribute to the recruitment of Tregs and promote immunosuppression after UVB exposure.

1.6 Mast cells

Mast cells derive from hematopoietic stem cells and acquire their mature form by migration of its precursors into various tissues, where they ultimately reside. They participate in a multitude of pathological and physiological processes by releasing various products upon exposure to diverse stimuli. Many of them initiate inflammation and tissue remodeling and are therefore regarded as proinflammatory products (Metz et al., 2007). Eliciting IgE-mediated Type 1 hypersensitivities such as allergic rhinitis (Bentley et al., 1992), atopic dermatitis (Kawakami et al., 2009), food allergies and asthma (Galli et al., 2008; Kraneveld et al., 2012; Sampson, 2004) represent probably the most prominent role for mast cells. However, it is known today, that mast cells not only act as promoters of inflammation, they also are key players in exerting immune tolerance.

Dermal mast cells are important in regulating skin homeostasis and display immune regulatory functions (Lu et al., 2006). Hart et al. demonstrated that dermal mast cells and mast cell-derived histamine are crucial for UVB-induced systemic immune suppression of CHS responses (Hart et al., 1998). A finding that was further substantiated by Byrne et al. showing that mast cells transmit the suppressive signal generated in mouse skin to the immune system by UV-induced migration of dermal mast cells to the draining lymph nodes which depicts a key event in the induction of immunosuppression (Byrne et al., 2008). This migration was dependent on expression of CXCR4 on mast cells, since its blocking by an antagonist diminished the migration of mast cells in the skin and a subsequent inhibition of UV-induced immune suppression.

Mast cells further on exert immunosuppressive functions after mosquito bites by downregulation of antigen-specific T cell responses, caused by mast cell-dependent IL-10 production (Depinay et al., 2006). Mast cell-derived carboxypeptidase and chymase have recently been reported to enhance murine resistance to the venoms of some species of snakes, honeybees, gila monsters and scorpions by degradation of the respective animal toxins (Akahoshi et al., 2011; Metz et al., 2006b; Rivera, 2006), whereupon it was long thought that

mast cell degranulation after envenomation would fuel the pathology mediated by the venom (Weisel-Eichler and Libersat, 2004).

Mast cell-deficient mice with a mutation in the *c-kit* region have become a powerful tool for investigating mast cells in many biological responses *in vivo*. $Kit^{W\text{-}Sh/W\text{-}Sh}$ mice have begun to be used more recently as a model to assess mast cell functions, since they display fewer phenotypic and developmental abnormalities, compared to models in which animals harbor other *c-kit* mutations (Grimbaldeston et al., 2005). They are neither anemic, nor sterile but the mutation results in an abolishment of mast cells soon after birth, together with melanocytes and interstitial cells of Cajal (Besmer et al., 1993; Duttlinger et al., 1993; Grimbaldeston et al., 2005).

Mast cells and Tregs interact with each other in different situations (Ganeshan and Bryce, 2012; Leavy, 2006; Piconese et al., 2009) and both have been shown to be critically dependent on each other in the maintenance of skin graft tolerance (Lu et al., 2006) as well as in a nephrotoxic serum nephritis model (Eller et al., 2011). In another murine model of glomerulonephritis (GN) missing mast cells led to decreased numbers of Tregs in lymph nodes and a reduced production of IL-10 which subsequently resulted in a more severe form of GN (Gan et al., 2012). Reconstitution with wild-type mast cells but not IL-10-deficient mast cells abated autoimmunity as well as severity of the disease.

Concerning PLE, Wolf et al. discovered that PLE patients exhibited decreased levels of papillary dermal mast cells that were restored after medical photohardening treatment (Wolf et al., 2014). The aforementioned mast cell-derived IL-10 might be of relevance, since it has the potential to prevent inflammatory leukocyte infiltration, thereby limiting the inflammation caused by chronic low dose UVB (Grimbaldeston et al., 2007) and recruits immune cells like Tregs that abrogate autoimmune responses (Lu et al., 2006; Singh et al., 2012, 2010). It still has to be investigated whether mast cells or its mediators contribute to the pathogenesis of PLE. All immunological mechanisms that might contribute to a formation of PLE are highlighted in Figure 2.

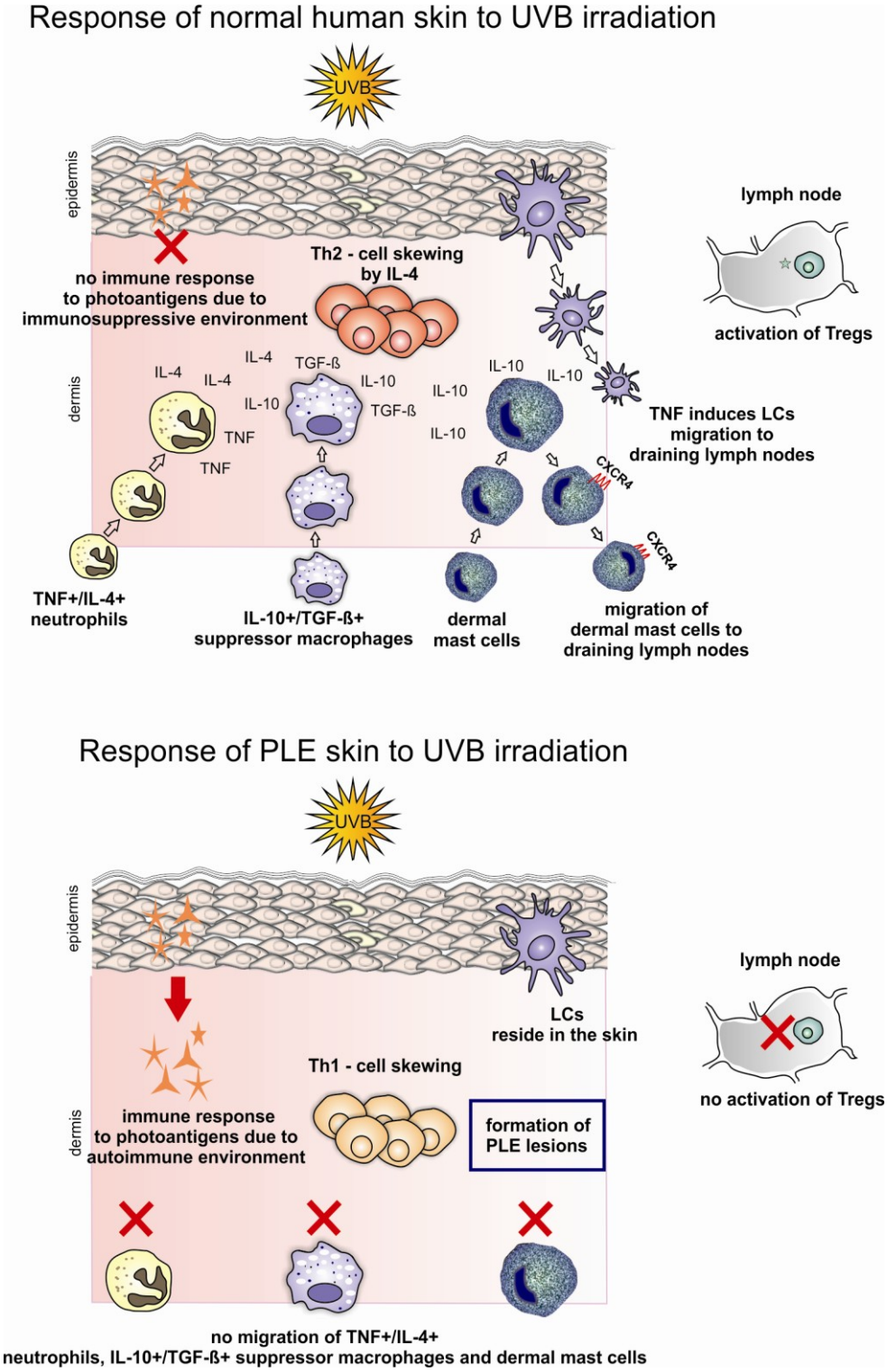

Figure 2. *Potential immunological mechanisms resulting in the pathomechanisms of PLE. Patients display an impaired infiltration of neutrophils, macrophages and mast cells upon UVB exposure. In healthy individuals LCs are activated by infiltrating immune cells and their mediators and migrate from the skin to the draining lymph nodes where they are thought to activate Tregs. In patients they reside in the skin and an immune response due to a change in*

immune environment is formed, leading to non-scarring, pruritic lesions of distinct morphology. Adapted from (Wolf et al., 2009) *and* (Gruber-Wackernagel et al., 2014).

2. STUDY PART 1: THE EFFECT OF NATURAL AND MEDICAL PHOTOHARDENING ON TREG LEVELS AND FUNCTION IN PLE PATIENTS

Aims

PLE is arising due to immunologic abnormalities that recur often on an annual basis each spring or early summer (Jansen, 1979). PLE patients are thought to be resistant to the immune suppressive properties of UV radiation and in the pathogenesis a delayed type hypersensitivity reaction is suggested directed against yet unidentified, endogenous antigens formed upon UV radiation (Wolf et al., 2009). Natural and medical photohardening leads to a "hardening effect" that ameliorates or entirely abrogates the symptoms of PLE. The exact immunological mechanisms behind natural and medical photohardening need to be determined. The aim of this thesis part was to investigate whether PLE patients display differences in peripheral Treg levels and/or function compared to healthy individuals, together with elucidating whether medical or natural photohardening has an influence on peripheral Treg levels and/or function.

Parts of **"The effect of natural and medical photohardening on Treg levels and function in PLE patients"** that are shown in the dissertation were published in the British Journal of Dermatology.

Levels and function of regulatory T cells in patients with polymorphic light eruption: relation to photohardening.

Schweintzger N, Gruber-Wackernagel A, Reginato E, Bambach I, Quehenberger F, Byrne SN, Wolf P.

Br J Dermatol. 2015 May 30. doi: 10.1111/bjd.13930.

2.1 MATERIAL AND METHODS

2.1.1 Study setup

This study was conducted at the Photodermatology Unit, Medical University of Graz, Austria to investigate the levels and function of Tregs in PLE patients and healthy controls (Cinical-Trials.Gov registration number NCT00555178). The following null and alternative hypotheses were tested: $H_0, < 30\%$ increase and $H_A, \geq 30\%$ increase in Treg levels comparing baseline with after phototherapy treatment. Sample size calculations were based on the data from a previous study (Miyara et al., 2005) and were performed using Power/Sample Size Calculator (Institute of Medical Statistics, Medical University Vienna, Austria) with an alpha error of 0.05 and a power of 0.8. Assuming a drop-out rate of 10% resulted in a group size of 23.

The inclusion criteria for the PLE patients were age above 18 years and good general health status. The diagnosis of PLE had to be confirmed by patient's history, histologic findings and⁄or phototesting procedures. Exclusion criteria included the presence or history of malignant skin tumours, dysplastic nevus syndrome, autoimmune diseases, systemic treatment with steroids or other immunosuppressive drugs (ongoing, within the last 6 months or planned during the study period), antinuclear antibodies (such as ds-DNA, Ro, La), specific immunotherapy (i.e. hyposensitisation treatment; ongoing, within the last 6 months or planned), and pregnancy or breast feeding. Patients seeking or not seeking photohardening therapy in spring were recruited. In addition, age-matched healthy control subjects were enrolled during the same periods of time. In the initial study protocol, patients with phototherapy-responsive diseases (such as psoriasis and atopic dermatitis) represented another study group. Two additional time points in late summer and fall were also part of the protocol for all patient groups. Due to substantial losses in patient follow-up numbers at those later time points together with large variability in the group of patients with phototherapy-responsiveness, this data has been omitted from the present analysis.

The study was approved by the local Ethical committee of the Medical University of Graz (No. 18-116 ex 06/07). All patients and controls gave informed consent and the study was conducted adherent to the Declaration of Helsinki principles.

2.1.2 Study subjects and phototherapy characteristics

Thirty PLE patients were enrolled in this study between 2008 and 2014. Standard 311nm UVB photohardening therapy (Gruber-Wackernagel et al., 2012; Wolf et al., 2013) was started in spring (immediately after the first blood collection; TP1, see below) in 23 of the patients (22 females and 1 male; mean age 37.4 years, range 18-75) 2–3 times per week for 4–9 weeks (median, 6 weeks). Seven PLE patients (all females; mean age 42.0 years, range 21-56) chose not to receive photohardening therapy and represented an additional study group (see Table 2.5.1 for details about patients and phototherapy). Nineteen healthy subjects (15 females and 4 males; mean age 38.6 years, range 24-61) without a history of PLE were enrolled and served as controls. One PLE patient not seeking phototherapy and one healthy volunteer dropped-out of the study after the first blood drawing, each of them due to personal reasons. Their data are not included in the patient demographics and study analysis (Fig.2.4.1).

2.1.3 Blood sample collection and processing

Blood was collected using lithium-heparin tubes (Vacuette®, Greiner Bio-One, Kremsmünster, Austria) before the start of photohardening in March to June and within 48 hours of the (pen)ultimate exposure of the photohardening treatment from May to August. The first time point (pre-photohardening) is labelled “TP1” and the second time point (post-photohardening) is labelled “TP2” in this report and its figures. The average time that elapsed between first and second blood withdrawal was 48.0 days (photohardened PLE group), 57.7 days (non-photohardened PLE group) and 55.4 days (controls). Blood materials were available from all patients for flow cytometry analysis and from a subset of 8 patients and 8 healthy controls for suppression assays and mRNA analysis.

2.1.4 PBMC isolation and flow cytometry

PBMCs were isolated by density gradient centrifugation using LymphoprepTM (Axis-Shield, Heidelberg, Germany) for processing and analysis by flow cytometry, RT-PCR and *in vitro* Treg suppression assays. The following antibodies were used for sorting of PBMCs using a FACSAria IIu cell sorter (BD Biosciences, San Jose, California): FITC-conjugated anti-human CD4 (clone RPA-T4), PE-Cy7-conjugated anti–human CD25 (M-A251), PE-conjugated anti–human CD127 (clone hIL-7R-M21), all from eBioscience (Vienna, Austria).

We used PE-conjugated anti-human CD127 (clone hIL-7R-M21), FITC-conjugated anti-human CD25 (clone 2A3), PerCP-conjugated anti-human CD4 (clone SK3) (BD Pharmingen, San Diego, CA) and APC-conjugated anti-human FoxP3 (clone PCH101) from eBioscience (Vienna, Austria) for quantification of human PBMCs. Data was acquired on a FACSCalibur flow cytometer and analysed with FlowJo software (version 7.6.5., Tree star, Inc., Ashland, Oregon, USA). All plots were pregated on $CD4^+$ lymhpocytes. The increase in percentage of Tregs as a proportion of either the CD4 subpopulation or the entire lymphocyte subpopulation was calculated by dividing TP2 by TP1.

2.1.5 Treg suppression assay

Treg suppression assays with blood samples from the subset of eight patients and eight control subjects were performed, as described (Singh et al., 2010). In brief, PBMCs were stimulated with 5µg/ml purified low endotoxin/ sodium azide-free mouse anti-human CD3 (clone UCHT1; BD Pharmingen, San Diego, CA) and 2.5µg/ml anti-human CD28 (Clone 28.2) (BioLegend, London, UK) for 96h. Cellular proliferation was measured with a Wallac 1450 MicroBeta® TriLux (Perkin Elmer, Brunn am Gebirge, Austria) via tritiated thymidine incorporation (1 µCi per [^{3}H]thymidine) (Amersham Biosciences, Piscataway, NJ) added for the final 16h of the 96h incubation. For analysis, the 1:1 ratio of T effector cells and Tregs was normalised to the proliferative rate of stimulated T effector cells alone (set to 100%).

2.1.6 RNA isolation and quantitative RT-PCR

RNA was isolated from PBMCs of a subset of PLE patients receiving phototherapy and healthy controls (n = 8 each) using the Qiagen fibrous mini kit (Qiagen, Hilden, Germany) and transcribed using First-Strand cDNA Synthesis kit (Roche, Basel, Switzerland). Quantitative RT-PCR was performed with primers specific for human *FoxP3* (forward: GCTCTGCACCTTCCCAAAT; reverse: TCTCTGGAGGAGACATTGTGC) and the *GAPDH* gene region as a control (forward and reverse primer, PPH00150E-200, Quiagen, Hilden, Germany). The reactions were run on a 7900HT Fast Real-Time PCR System (Life Technologies, Vienna, Austria) using RT^2 SYBR Green-qPCR Master Mix (Qiagen, Hilden, Germany). To normalise transcripts to *GAPDH* and to calibrate the fold change the ΔΔCt method was used. Data are presented as fold change comparing TP2 to TP1.

2.1.7 Statistical analysis

Statistical differences were determined by using Kruskal-Wallis test, Wilcoxon signed rank test or Mann-Whitney U test, as appropriate for the data. Data presented are expressed as means ± SEM or means with 95% confidence intervals. All analyses were performed with Prism 5.0 (GraphPad software Inc., SD, California) or R 3.1.2 (www.r-project.org). Statistical significance was set at $P< 0.05$.

2.2 RESULTS

2.2.1 Treg numbers are increased in PLE patients after photohardening treatment

To study the role of Tregs in PLE, their numbers were investigated by staining PBMCs of patients and controls for CD4, CD25, CD127 and FoxP3 before (Fig.2.4.2a) and after photohardening treatment (Fig.2.4.2b). Flow cytometry analysis of PBMC from PLE patients before (TP1) and after (TP2) medical photohardening revealed a significant increase in the mean percentage of $CD4^{+}CD25^{+}$ $FoxP3^{+}$ Tregs in both the CD4+ subpopulation (1.56% vs. 2.11% [an increase of 35.3%]; $P = 0.0049$) and the total lymphocyte population (0.44% vs. 0.57% [an increase of 29.5%]; $P = 0.0049$) (Fig. 2.4.3a and for descriptive statistics see Table 2.5.2). There was no statistically significant change in the number of Tregs in the other two patient groups when comparing TP1 with TP2 (Fig.2.4.3a and Table 2.5.2). Changes in Treg numbers for the three different patient groups are depicted in Fig.2.4.3b. These were calculated by plotting the mean ratio and 95% CI between TP2 and TP1 for PLE patients with phototherapy, PLE patients without phototherapy and healthy control subjects. Any number above 1 is representative of an increase in Tregs during the time course. The only ratio to show a significant increase was that for PLE patients undergoing photohardening therapy with 1.37 (95% CI, 1.12-1.68; $P = 0.0027$) as a proportion of CD4+ T cells, and 1.50 (95% CI, 1.21-1.85; $P = 0.00059$) as a proportion of all lymphocytes. However, a Kruskal-Wallis test revealed no significant differences in Treg levels among the three groups at baseline (TP1) and at TP2. There were no significant differences in total leucocyte or lymphocyte numbers comparing the PLE patients with healthy control subjects either at TP1 or TP2 or between the two time points (data not shown).

2.2.2 Treg function is significantly impaired during spring in PLE patients

The Treg function was also investigated in a subset of eight patients receiving medical photohardening and eight healthy controls subjects by measuring their capacity to inhibit T cell proliferation. These number of patients and control subjects were representative since similar courses of Treg levels in both subpopulations were observed comparing pre -vs. post - phototherapy values as displayed in the entire groups, determined by flow cytometry, (i.e., an increase in PLE patients and no significant change in control subjects; data not shown). Tregs from healthy controls significantly suppressed the proliferation of CD3/CD28-stimulated ef-

fector T cells (Teff) at TP1 by 59% (P = 0.0156) (100% proliferation at 0:1 ratio of Teff compared to 41% proliferation at a 1:1 ratio; Fig.2.4.4b). In contrast, Tregs from PLE patients at this time point lacked any capacity to suppress Teff proliferation (Fig.2.4.4a). No significant suppression of Teff proliferation was observed at TP2 in either group of subjects. However, when the change in suppressive activity over the time course was analysed an increase in Treg-mediated suppression in PLE patients from 66% to 115% was displayed. In contrast, suppressive activity over the same time course for healthy controls *decreased* from 65% to 41%. For comparison, this was expressed as a ratio of the mean Treg suppression at TP2 with that at TP1 (Fig.2.4.4c). Ratios below 1 indicate a rise in suppressive activity whereas ratios above 1 indicate a fall. Analysis of individual ratios confirmed a trend towards difference between patients receiving 311nm UVB and healthy controls, with a mean TP2/TP1 ratio of 0.97 and 2.63 respectively (P = 0.084; Fig.2.4.4c).

2.2.3 *FoxP3* mRNA levels are significantly upregulated in PLE patients after phototherapy

Because higher expression levels of FoxP3 correlates with enhanced Treg suppressive capacity (Sakaguchi et al., 2010), it was investigated whether medical photohardening therapy affects *FoxP3* mRNA levels in PLE patients by qRT-PCR. Transcripts were normalized to *GAPDH* and compared to TP1 for PLE patients and controls (the same subset of patient and control groups were used as shown in Fig.2.4.4c; one patient and one healthy control subject had RNA yields that were too low to be included in this analysis). Relative *FoxP3* mRNA levels were up-regulated after photohardening in six of seven PLE patients (Fig.2.4.5). In contrast, the amount of *FoxP3* mRNA decreased in five of seven healthy individuals between the two time points. This was a statistically significant difference between the two groups (P=0.0083).

2.3 DISCUSSION

Photohardening therapy given to PLE patients in spring enables them to tolerate their first high dose of sunlight later in the season with minimal or no eruption. Evidence was herewith provided that despite having the same numbers of circulating Tregs as control subjects at the start of spring, Tregs of PLE patients have an impaired suppressive function (Fig.2.4.4a). This implicates Tregs in the pathogenesis of PLE and potentially explains why these patients are resistant to the normal immune suppressive properties of UV. Because activation of Tregs is a major way in which UV suppresses cutaneous immunity, it was hypothesised that successful UV-induced therapeutic hardening was efficacious, in part, by boosting Treg numbers and/or function. Supporting this hypothesis, it was discovered that medical photohardening with 311nm UVB not only significantly increased the numbers of Tregs by more than a third (Fig.2.4.3a and Table 2.5.2), but also increased Treg expression of *FoxP3* mRNA, an indicator of increased suppressive function (Fig.2.4.5). A similar increase in the level of Tregs, though not statistically significant, was also observed in patients not receiving medical phototherapy (Fig.2.4.3). This is most likely to have occurred through hardening by exposure to natural sunlight. Alternatively, a seasonal effect independent of UV exposure may have caused these changes in both groups of patients compared to healthy control subjects. A limitation of this study is the unequal group size of PLE patients and further, that the number of enrolled patients without phototherapy was rather low.

The precise reason why PLE patients have normal numbers of Tregs with sub-optimal suppressor capabilities is unknown and the UV-induced cutaneous signal that normalises UV-immune suppression in PLE remains to be determined. One possibility is that genetic factors might play a role (McGregor et al., 2000; Millard et al., 2001a, 2001b, 2001c, 2000). Genetic regulation might alter the normal response to UV irradiation in patients through modulation of the immune suppressive function of patients directly by affecting Tregs or indirectly by affecting cells or mediators that recruit or activate Tregs.

For instance, the glutathione-S-transferase (GST) which is an enzyme that detoxifies reactive oxygen species (ROS) was reported to exert a possible protective effect against PLE since the carrier frequency of the GSTP1 allele was found to be lower in patients than in controls, which would support an involvement of ROS in the pathogenesis of the disease (Millard et al., 2008). This was in contrast to another study that could not find an association between the GST gene family and PLE (Zirbs et al., 2013). Guarrera and Rebora investigated the

hydrosoluble antioxidant capacity which included uric acid, bilirubin, vitamin C, thyols and glutathione and found decreased rates in PLE patients and moreover lower values in female patients and controls compared to males, which increased in diseased women with age (Guarrera and Rebora, 2007). That PLE has a disproportionately higher incidence in young women is known, but the reason is unclear. Sex hormones like 17beta-estradiol prevent UV-induced suppression of contact hypersensitivities through limiting IL-10 secretion from keratinocytes (Aubin, 2004; Hiramoto et al., 2004). This is substantiated by a study of Widyarini et al (Widyarini et al., 2006) who demonstrated a natural photoimmunoprotective role of the estrogen receptor, since blocking of it exacerbated the immune suppression from solar-simulated UV-radiation. Further studies are required to investigate a possible gene link to gender influence which certainly may be not solely responsible for the pathogenesis of PLE, since the control group of this report was well matched and mostly comprised of women, did exhibit a differential Treg regulation.

Studies with murine models showed that dermal mast cell density determines ones susceptibility to UV immune suppression (Hart et al., 1998). This in turn is dependent on the migration of mast cells into and away from the skin to activate suppressor cells in the local draining lymph nodes (Byrne et al., 2008). While the precise mechanism by which dermal mast cells mediate UV-induced immune suppression is not known, their production of immunoregulatory IL-10 following UV is involved (Chacón-Salinas et al., 2011; Grimbaldeston et al., 2007). Alternatively, mast cells might activate Tregs that are required to maintain peripheral tolerance (Lu et al., 2006; Schweintzger et al., 2015a). Intriguingly, an observation was made recently where photohardening increased the numbers of mast cells in the papillary dermis of PLE patients (Wolf et al., 2014). This was accompanied by a recovery in neutrophil responsiveness to the chemo attractants leukotriene B4 and formyl-methionyl-leucyl-phenylalanin (Gruber-Wackernagel et al., 2011b). That PLE is linked to low numbers of Tregs is supported by a recent observation by Gambichler et al. (Gambichler et al., 2013), showing a low Treg infiltration together with a decreased expression of the immunoregulatory factors TGF-β1, IL-10 and RANKL in UVA1-exposed skin lesions of PLE patients. Furthermore, Gruber-Wackernagel et al. (Gruber-Wackernagel et al., 2011a) have shown that lesional skin from PLE patients contained relatively low numbers of $Foxp3^+$ cells in the dermal infiltrate compared to calcipotriol-pretreated skin that showed less clinical PLE severity. The observation that 311nm UVB increased Treg levels in PLE patients is consistent with the results of a recent study in patients with various skin diseases who showed an increase in peripheral Tregs after treatment with 311nm UVB (Milliken et al., 2012). The mechanism by which UV

photohardening increases the level and function of Tregs may involve the production of vitamin D. Indeed, its supplementation has been shown to be associated with significantly increased numbers of Tregs in apparently healthy individuals (Bock et al., 2011; Prietl et al., 2010). Said so, a previous study (Gruber-Wackernagel et al., 2012) has indicated that 311nm UVB hardening was capable of increasing serum 25-hydroxyvitamin-D3 levels which may be low in patients with photosensitivity and cases of photodermatoses, including PLE (Lim and Snauwaert, 2014; Reid et al., 2012; Rhodes et al., 2014).

These results suggest that a decreased function of Tregs might play a role in the formation of PLE and an increase in the number of Tregs might be a compensatory mechanism by which the immune system intends to counteract the susceptibility to PLE formation.

2.4 FIGURES

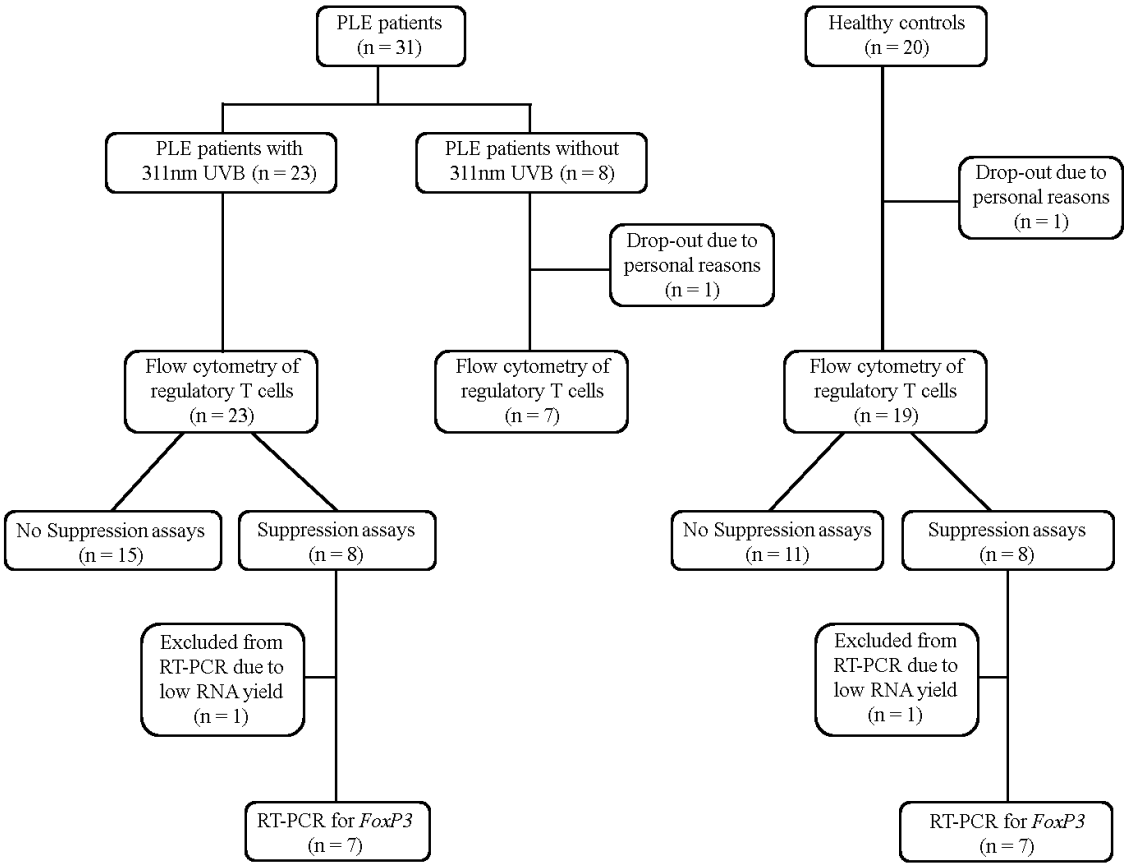

Figure 2.4.1 Flow diagram showing numbers of PLE patients and healthy controls at each stage of the study.

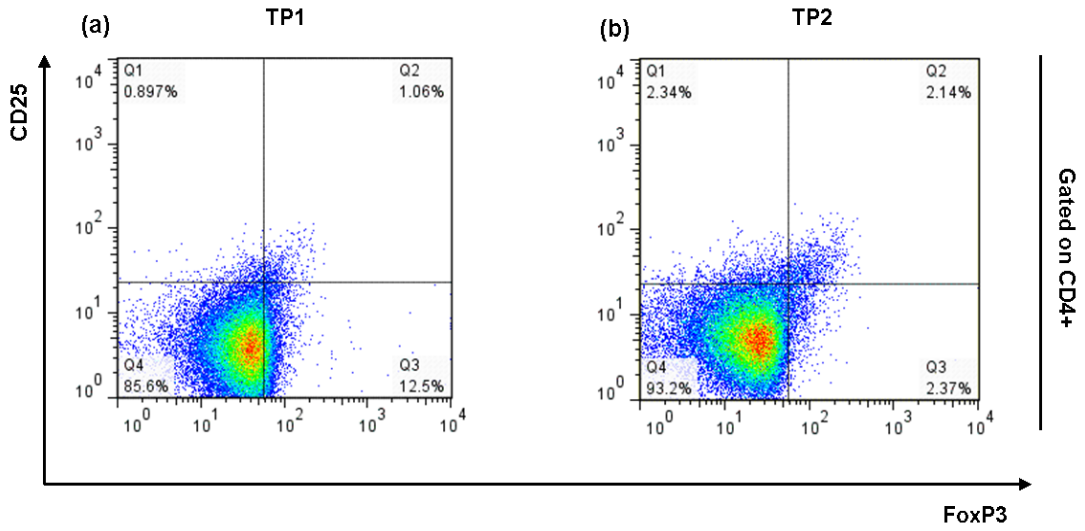

Figure 2.4.2 PBMCs of patients and healthy controls were stained with antibodies for CD4, CD127, CD25 and FoxP3. Cells were pregated on CD4+ for the analysis. Phenotypic analysis of peripheral Tregs from a PLE patient receiving 311nm UVB at (a) TP1 and (b) TP2 by flow cytometry.

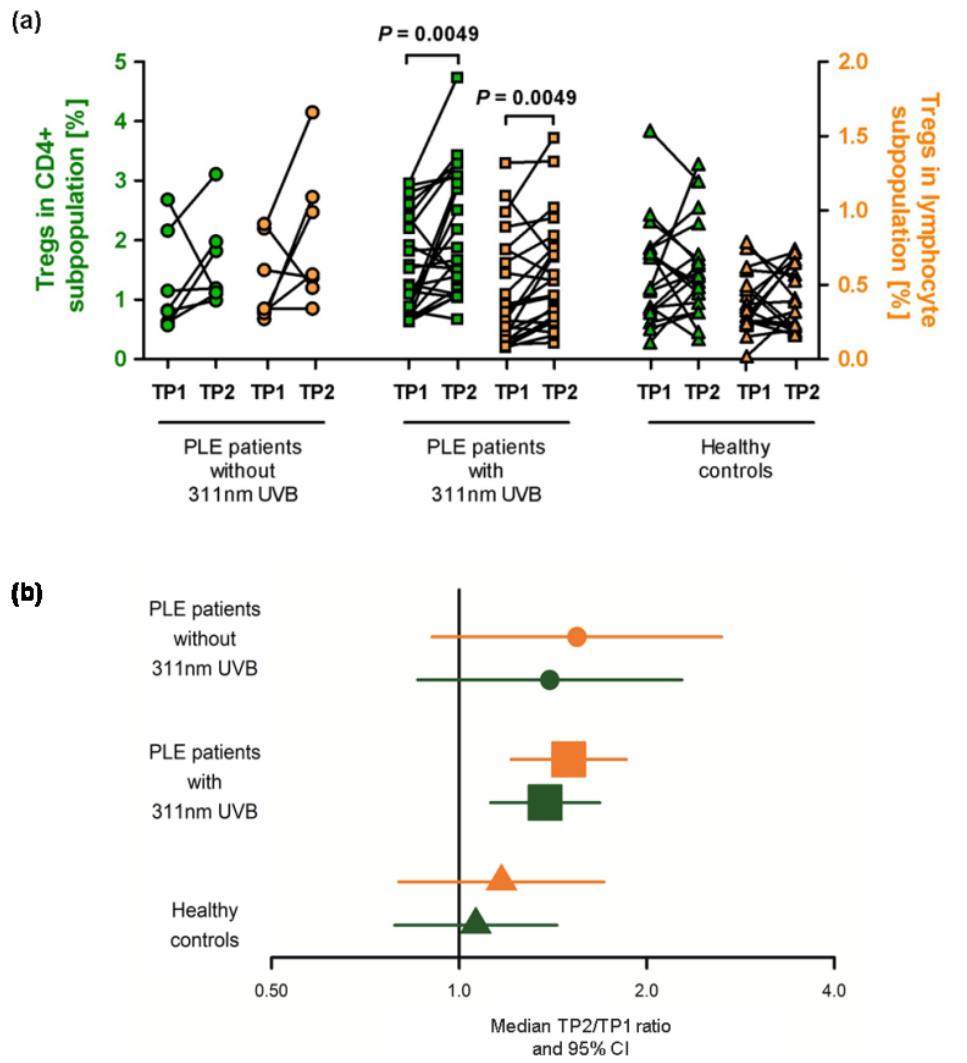

Figure 2.4.3. Photohardening treatment increases the number of Tregs in PLE patients. PBMCs of patients and healthy controls were stained with antibodies for CD4, CD127, CD25 and FoxP3. (a) Percentages of Tregs as a proportion of CD4+ cells (green symbols) or all lymphocytes (orange symbols) in PLE patients with or without 311nm UVB compared to healthy individuals at TP1 and TP2. (b) Mean TP2/TP1 ratio (± 95% CI) for the percentage of Tregs in CD4+ (green symbols) and lymphocyte (orange symbols) subpopulations from each of the three subject groups. Data sets were logarithmised before calculation of ratios. n = 7, patients without 311nm UVB; n = 23, patients with 311nm UVB and n = 19, healthy controls. *P*-values were determined by Wilcoxon test.

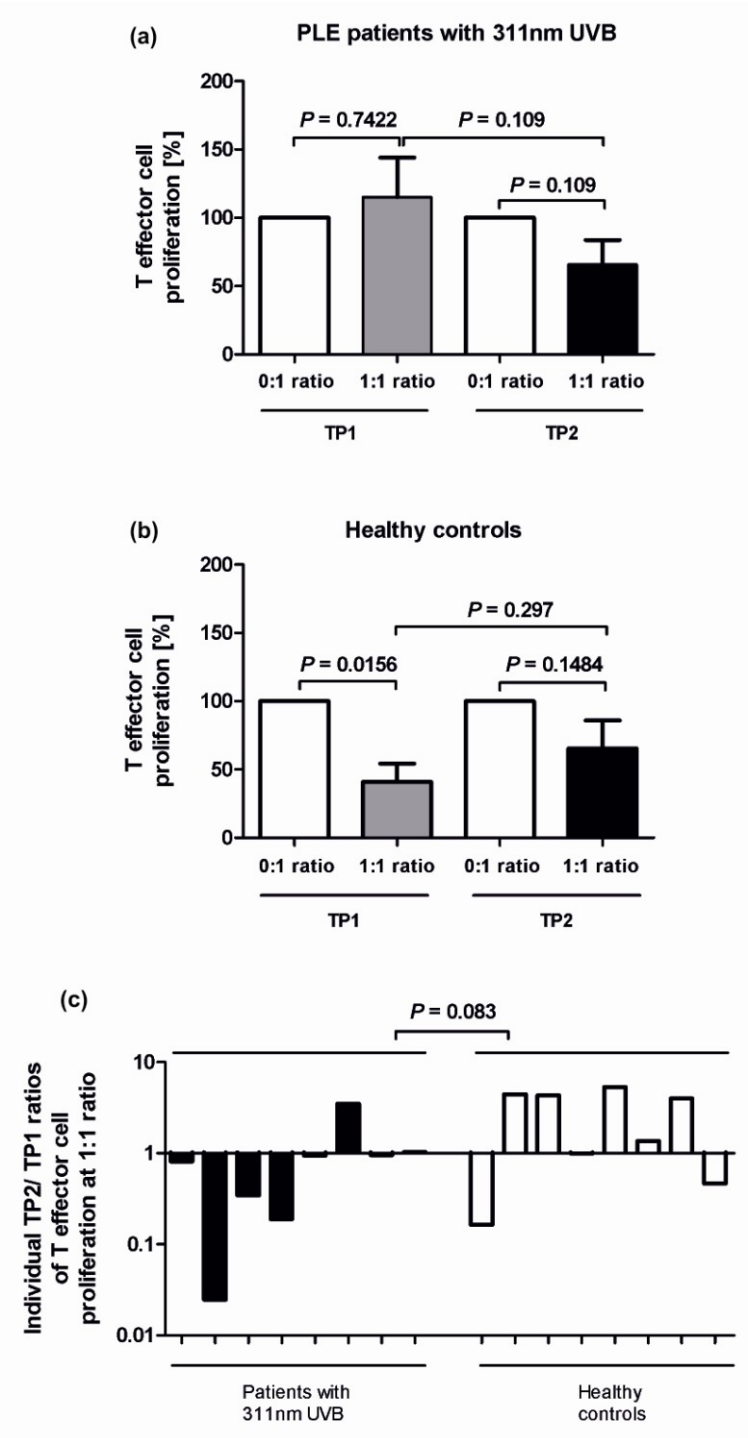

Figure 2.4.4. Tregs from PLE patients have a reduced immune suppressive capacity. Treg suppression assays from (a) PLE patients and (b) healthy controls at TP1 and TP2. Data shown are mean ± SEM proliferation rates, normalised to effector cell proliferation alone (0:1 ratio = 100%) for PLE patients and healthy controls. 1:1 ratio represents the results of co-culture of the same number of T effector (CD4+CD25-CD127+) and Tregs (CD4+CD25+CD127-). (c) Individual TP2:TP1 ratios of T effector cell proliferation. n = 8,

PLE patients; n = 8, healthy controls. (a,b) *P*-values were determined by Wilcoxon test and (c) Mann Whitney U test.

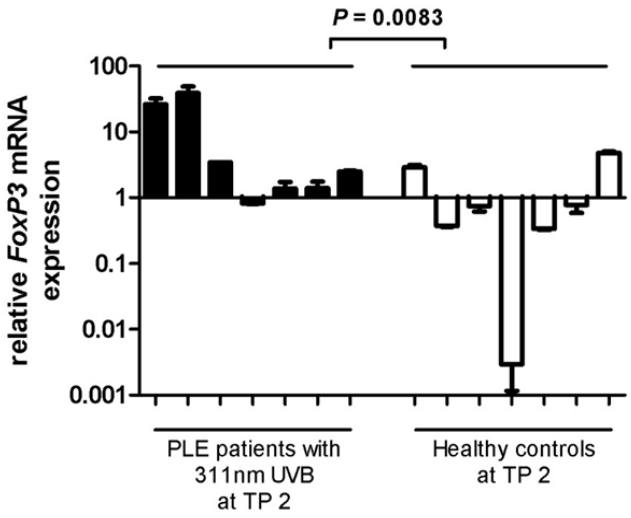

Figure 2.4.5 311nm UVB upregulates *FoxP3* mRNA in PLE patients. RT-PCR results from PBMCs of patients and healthy controls are shown as fold change of mRNA (TP2 - TP1) (mean ± SEM). The ΔΔCt method was used to normalise transcripts to *GAPDH*. n = 7, patients with 311nm UVB and n = 7, healthy controls. *P*-values were determined by Mann Whitney-test.

2.5 TABLES

Table 2.5.1. Characteristics of PLE patients.

Characteristics	Patients without 311nm UVB	Patients with 311nm UVB
Gender	f: 7; m: 0	f: 22; m: 1
Age in years (range)	42 (21-56)	37.4 (18-75)
Mean disease duration in years (range)	14 (2-35)	10 (1-30)
Skin phototype*, n	II: 1; III: 6	II: 5; III: 18
Pathology of PLE lesions	**m: 1; mp: 4; pap: 1; pv: 1	m: 0; mp: 9; pap: 11; pv: 3
Predilection body site	V-neck: 7; arm: 3; forearm: 4; thighs: 3; abd.: 2	V-neck: 21; arm: 12; forearm: 7; thighs: 7
Mean no. of exposures (range)	none	17.6 (11-20)
Mean weeks of treatment (range)	none	6.4 (4-9)
Mean starting dose in J/cm^2 (range)	none	0.3 (0.2-0.5)
Mean total dose in J/cm^2 (range)	none	13.5(5.76-20.6)

* According to Fitzpatrick classification; ** m = macular; mp = maculopapular; pap = papular; pv = papulovesicular; abd. = abdomen

Table 2.5.2. Percentages of $CD4^{+}CD25^{high}CD127^{-}FoxP3^{+}$ Tregs in PLE patients and healthy controls, as assessed by flow cytometry.

Type of subpopulation	PLE patients								Healthy controls			
	without 311nm UVB				with 311nm UVB							
	time points		percent increase	p-value	time points		percent increase	p-value	time points		percent increase	p-value
	TP 1	TP 2			TP 1	TP 2			TP 1	TP 2		
	mean percentage (95% CI)				mean percentage (95% CI)				mean percentage (95% CI)			
Tregs of CD4+ cells	1.26 (0.49-2.03)	1.62 (0.91-2.32)	28.6	0.297	1.56 (1.21-1.91)	2.11 (1.65-2.56)	35.3	**0.0049**	1.46 (1.05-1.87)	1.51 (1.13-1.89)	3.4	0.983
Tregs of total lymphocytes	0.52 (0.26-0.78)	0.81 (0.38-1.24)	55.8	0.219	0.44 (0.28-0.60)	0.57 (0.41-0.73)	29.5	**0.0049**	0.38 (0.28-0.47)	0.41 (0.31-0.51)	7.9	0.587

P-values result from comparing TP1 vs. TP2 values by Wilcoxon test.

3. STUDY PART 2: INFLUENCE OF SEASON ON VITAMIN D LEVELS, TREG NUMBERS AND TREG FUNCTION IN PLE PATIENTS

Aims

Since it was observed in the first part of the thesis that PLE patients not receiving medical photohardening therapy also showed an increase in Treg numbers (Schweintzger et al., 2015b), it can be speculated that natural hardening from sunlight might have contributed or caused this effect. Said so, a recent study in healthy individuals has shown that oral vitamin D supplementation resulted in increased Treg numbers (Prietl et al., 2010). Therefore, the aim of this thesis part was to investigate whether the progressing season leads to changes in Treg numbers and function in PLE patients, by following patients from January to June of a season from 2014 to 2015. The possible relation of vitamin D, Treg levels and Treg function was additionally investigated.

3.1 MATERIALS AND METHODS

3.1.1 Study design

This study was originally set up at the Photodermatology Unit, Medical University of Graz, Austria as a randomized, double-blinded placebo-controlled trail to assess the effect of oral vitamin D supplementation on susceptibility to disease manifestation in patients with a history of PLE. The study was approved by the local Ethical committee of the Medical University of Graz with the EudraCT-Nr.: 2012-000300-15. All patients provided written informed consent and the study was conducted adherent to the Declaration of Helsinki principles. Key-eligibility criteria were diagnosis of PLE which had to be confirmed by patient's history, phototesting procedures and⁄or histologic findings, age above 18 years and good general health status. Exclusion criteria included intolerance to the vitamin D study medication (Oleovit D_3^{TM}) or its solvent (coconut oil), presence or history of malignant skin tumours, dysplastic nevus syndrome, photosensitive diseases, sarcoidosis, renal insufficiency, topical or oral treatment with vitamin D within 3 or 6 months, antinuclear antibodies (anti-ds-DNA, anti-Ro/La), 25-hydroxy vitamin D levels > 30ng/ml at the screening visit, hypercalcaemia > 2,65 nmol/L in serum, ongoing treatment with thiazide diuretics or glycosids, systemic treatment with steroids or other immunosuppressive drugs within 4 weeks and direct UV radiation of skin sites to be tested within 8 weeks before trial start.

3.1.2 Study subjects and procedures

Twenty-four PLE patients (19 females and 5 males; mean age 46.3 years, range 25-76) were recruited between 2012 and 2014. The recruitment time point is labelled as "TP1" in this part of the dissertation, where 25-hydroxy vitamin D (25(OH)D) serum levels were determined. Sixteen of this patients (66%) displayed normal vitamin D serum levels (>30 ng/ml) and only eight patients (33%) were identified with low 25(OH)D serum levels (<30ng/ml) and were subjected (according to the study protocol) to the administration of oral vitamin D or placebo (see Table 3.5.1 for patients characteristics). This part of the dissertation was therefore focused on investigating a possible influence of season on Treg numbers, Treg function and vitamin D serum levels in 24 PLE patients at TP1, spanning from January to June, whereas the other time points were omitted from the present analysis (the original study setup is shown in Table 3.5.2). Two prescreened patients were not enrolled in the study. One patient was diag-

nosed with lupus erythematosus and one patient withdrew from the trial before any study procedure was done. Their data are not included in the patient demographics and study analysis (see Fig.3.4.1 for flow diagram of patients).

3.1.3 Blood sample collection

Blood was collected for flow cytometry and Treg suppression assays using lithium-heparin tubes (Vacuette®, Greiner Bio-One, Kremsmünster, Austria). Blood for 25(OH)D serum level determination was collected in serum tubes (Vacuette®, Greiner Bio-One, Kremsmünster, Austria).

3.1.4 Quantitative 25-hydroxyvitamin D_3 determination

25-hydroxyvitamin D_3 (25(OH)D) serum level determination was performed with the fully automated, quantitative, chemiluminescent immunoassays IDS-iSYS 25-Hydroxy Vitamin D and IDS-iSYS 25-Hydroxy Vitamin D^S with a IDS-iSYS 25OHD Control Set on the IDS-iSYS Multi-Discipline Automated Analyzer (IDS plc, Boldon, UK) at the Department of Internal Medicine, Division of Endocrinology and Metabolism, Medical University of Graz, Austria.

3.1.5 PBMC sorting and flow cytometry

For Treg suppression assays PBMCs were isolated by density gradient centrifugation using Lymphoprep^{TM} (StemCell Technology, Grenoble, France). The antibodies used for sorting and flow cytometry have been previously published (Schweintzger et al., 2015b). For flow cytometry of Tregs all plots were pregated on $CD4^+$ lymhpocytes. Calculation of absolute Tregs numbers is based on the Tregs in the leukocyte subpopulation and the absolute numbers of leukocytes given as Giga per Liter (G/L) from the blood count determined with the Sysmex XE-2100 (Sysmex Co., Kobe, Japan).

3.1.6 Treg suppression assays

Treg suppression assays were performed, as described (Schweintzger et al., 2015b; Singh et al., 2010). In brief, PBMCs were isolated with Lymphoprep^{TM} (StemCell Technology, Greno-

ble, France) and stimulated with 5µg/ml purified, plate-bound, low endotoxin/ sodium azide-free mouse anti-human CD3 (clone UCHT1; BD Pharmingen, San Diego, CA) and 2.5µg/ml anti-human CD28 (Clone 28.2) (BioLegend, London, UK) for 96h. Cell proliferation was visualized via tritiated thymidine incorporation (1 µCi per [^{3}H]thymidine) (Amersham Biosciences, Piscataway, NJ) added for the final 16h of the 96h incubation with a Wallac 1450 MicroBeta® TriLux (Perkin Elmer, Brunn am Gebirge, Austria). For analysis, the 1:1 ratio (co-culture of the same number of T effector cells and Tregs) was normalised to the proliferative rate of stimulated T effector cells alone (set to 100%) to determine the suppressive capacity of Tregs.

3.1.7 Statistical analysis

Statistical analyses were done using Spearman correlation, Mann-Whitney U test and Wilcoxon signed rank test, as appropriate for the data, with Prism 5.0 (GraphPad software Inc., SD, California) or R 3.1.2 (www.r-project.org). Statistical significance was set at $P< 0.05$.

3.2 RESULTS

3.2.1 Relative and absolute Treg numbers display a seasonal tendency towards an increase

To study the role of season at TP1 (plotted as days of year in the graphs, spanning from January to June) on Tregs of PLE patients, their numbers were investigated by staining PBMCs for CD4, CD25, CD127 and FoxP3 as described in a previous study (Schweintzger et al., 2015b). Flow cytometry analysis of PBMCs from PLE patients revealed a statistically non-significant correlation between CD4+CD25+FoxP3+ Treg numbers and day of year for both the CD4+ subpopulation (Fig.3.4.1a) and the total lymphocyte subpopulation (Fig.3.4.1b). Absolute Treg numbers showed also a statistically non-significant association to days of year (Fig.3.4.3a). A cut off at day 100 was performed and relative and absolute Treg numbers from day 10 to 42 were compared to relative and absolute Treg numbers from day 108 to 176. By doing so, a significant increase in percent of Tregs of the CD4+ subpopulation from day 108 to 176 was detected ($P = 0.0466$; Mann-Whitney testing), that was mirrored by an increase in absolute numbers of Tregs ($P = 0.0297$). Using the observed standard deviation (SD) from the eight patients measured in the period from January to April and the 16 patients measured in the period from May to June posthoc power analysis revealed that the percentage of Tregs in lymphocyte population should have differed by 110% (SD = 0.59) and the CD4 subpopulation by 100% (SD = 1.20) in order to achieve 80% power with an alpha error of 0.05.

The possible influence of season on the suppressive Treg function of PLE patients was investigated, measured via suppressive assays. The T effector cell populations of six patients did not show sufficient proliferation upon stimulation with CD3/CD28 antibodies. (i.e. 10.000 corrected counts per million, CCPM1) at TP1 and were omitted from this analysis. In addition, six patients displayed a high variability in the duplicate measurements in the proliferation of Teff cells or the 1:1 coculture (i.e. greater than 50% difference between the two measurements) and were also excluded from the present analysis. No statistically significant correlation was found between Treg suppressive capacity and day of year (Fig.3.4.3b). Absolute numbers of other leukocytes (monocytes, lymphocytes, neutrophil and eosinophil granulocytes) determined from PLE patients at TP1 showed also no relationship to day of year (data not shown).

3.2.2 Individual 25(OH)D serum levels of PLE patients are influenced by season.

When correlating 25(OH)D serum levels of PLE patients measured at TP1 with day of year a statistically significant direct relationship was found (Spearman R = 0,586; Spearman P = 0,0026), as 25(OH)D serum levels were increasing towards June (Fig.3.4.4a). No relationship between absolute Treg numbers and 25(OH)D serum levels from PLE patients was observed when correlating these two parameters by Spearman testing (Fig.3.4.4b).

3.2.3 No differences in Treg numbers or function was found between PLE patients with low or normal 25(OH)D serum levels.

The absolute Treg numbers at TP1 of eight patients displaying low 25(OH)D levels (<30ng/ml) were compared to numbers of patients displaying normal 25(OH)D levels. The absolute Treg numbers displayed no significant difference between patients with low and patients with normal 25(OH)D levels (Fig.3.4.5a). Relative Treg numbers also revealed no differences (data not shown). The suppressive activities of Tregs from patients with low and patients with normal 25(OH)D levels did also not differ (Fig.3.4.5b). Additionally, correlating Treg function of patients with their respective 25(OH)D levels failed to display a relationship (Fig.3.4.5c).

3.3 DISCUSSION

The seasonal influence on vitamin D serum levels, Treg numbers and Treg function of PLE patients was investigated in this part of the thesis. In a previous study 311nm UVB photohardening resulted in a significant increase in Treg numbers of PLE patients. The study also showed a non-significant increase in patients not subjected to photohardening therapy. It was speculated that the effect seen in the latter patients may have been due to photohardening by natural sunlight (Schweintzger et al., 2015b). Therefore a possible effect of season on Treg numbers and Treg function of PLE patients focusing on TP1, spanning from January to June was investigated. No significant correlation of percentages of Treg in the CD4+ or the lymphocyte subpopulation with day of year was detected (Fig.3.4.2a and b), which could also not be observed when absolute Treg numbers were plotted (Fig.3.4.3a). However a tendency towards an increase in relative (Fig.3.4.2.a and b) as well as absolute Treg numbers (Fig.3.4.3a) was found towards June which was substantiated by a cut off analysis (see 3.2.1 results section). A significant relationship of day of year with Treg function of PLE patients measured at TP1 could not be detected (Fig.3.4.3b). This is consistent with the results of a previous study (Schweintzger et al., 2015b).

In this part of the thesis 25(OH)D serum levels from PLE patients displayed a positive correlation with days of year at TP1, showing an increase towards June (Fig.3.4.4a). However a direct correlation of 25(OH)D levels with absolute Treg numbers was not observed (Fig.3.4.4b). When relative Treg numbers of PLE patients were correlated to 25(OH)D levels no relationship could be found either (data not shown). Additionally, absolute Treg numbers and Treg function of PLE patients with low 25(OH)D serum levels were compared to PLE patients with normal serum levels at TP1 and no significant differences were observed (Fig.3.4.5a and b). Moreover, no difference in Treg function between the two groups of patients was present (Fig.3.4.5c).

Together this indicates that Treg numbers and Treg suppressive function in PLE patients is independent from 25(OH)D serum levels. This is consistent with the results of the study by Smolders et al. (Smolders et al., 2009) who found no correlation between 25(OH)D levels and number of Treg in multiple sclerosis patients. This contrasts the results of the study by Bock et al. (Bock et al., 2011) where oral vitamin D supplementation resulted in an increase in Treg

numbers in apparently healthy individuals. Thus, at least in PLE patients an effect of UV radiation on Treg numbers and function may arise through non vitamin D-dependent pathways. Alternatively, other factors may contribute to the affection of Treg during the season. These factors include other components of sunlight like visible light (Choi et al., 2012; Roberts, 1995) and infrared radiation (Danno and Sugie, 1996; Muili et al., 2013), temperatures and length of daytime hours (Mann et al., 2000; Nelson and Demas, 1997; Song et al., 2015; Srinivasan et al., 2008).

Why PLE patients display low Treg levels that are also impaired in function is still unknown (see study part 1 of the thesis), but genetic factors (McGregor et al., 2000; Millard et al., 2001b, 2001c, 2000) or sex hormones (Aubin, 2004; Widyarini et al., 2006) may be involved which might lead to a lack of UV-induced immunosuppression. It is hypothesized that this absence results in the development of delayed type hypersensitivity reactions in PLE patients with immune responses directed against unidentified, endogenous antigens that are formed upon UV radiation (Norris et al., 1989). This is substantiated by the study of Koulu et al. (Koulu et al., 2010) finding an impaired state of immunotolerance in PLE patients, since only 8% of PLE patients tested (1 out of 13) showed a long-term immunotolerance against a contact allergen when immunosuppressed by UV radiation.

However, no definite conclusion can be drawn out of the data due to high variability and relatively low patient numbers, as evidenced by the posthoc power analysis presented in section 3.2.1.

3.4 FIGURES

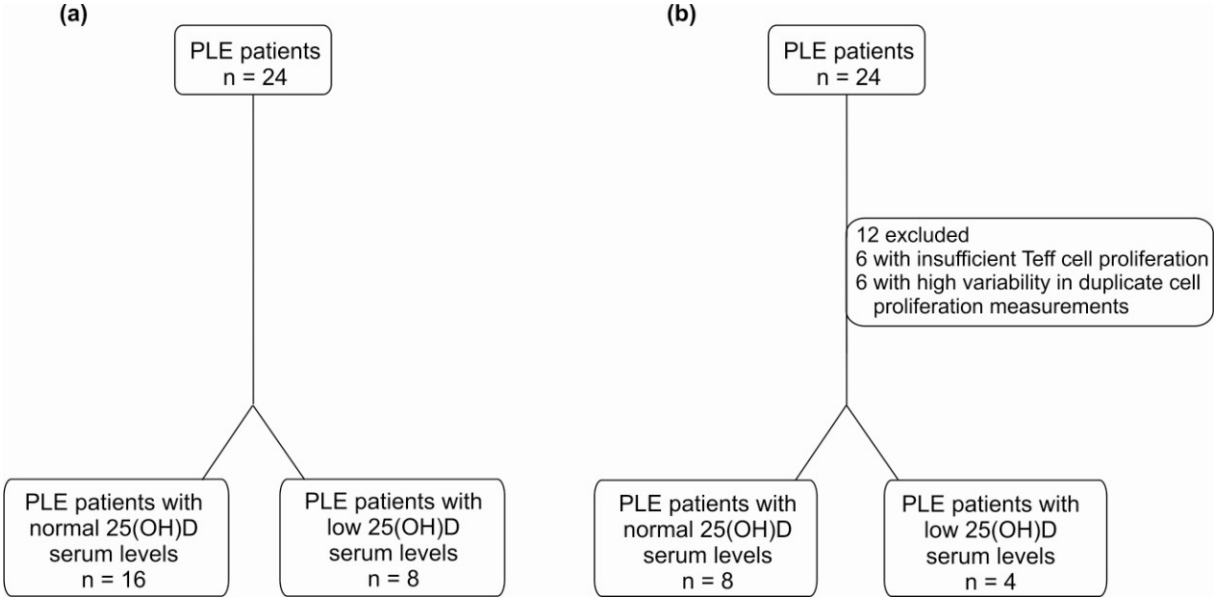

Figure 3.4.1. Flow diagram showing numbers of PLE patients at TP1 (a) flow cytometry (b) Treg suppression assays.

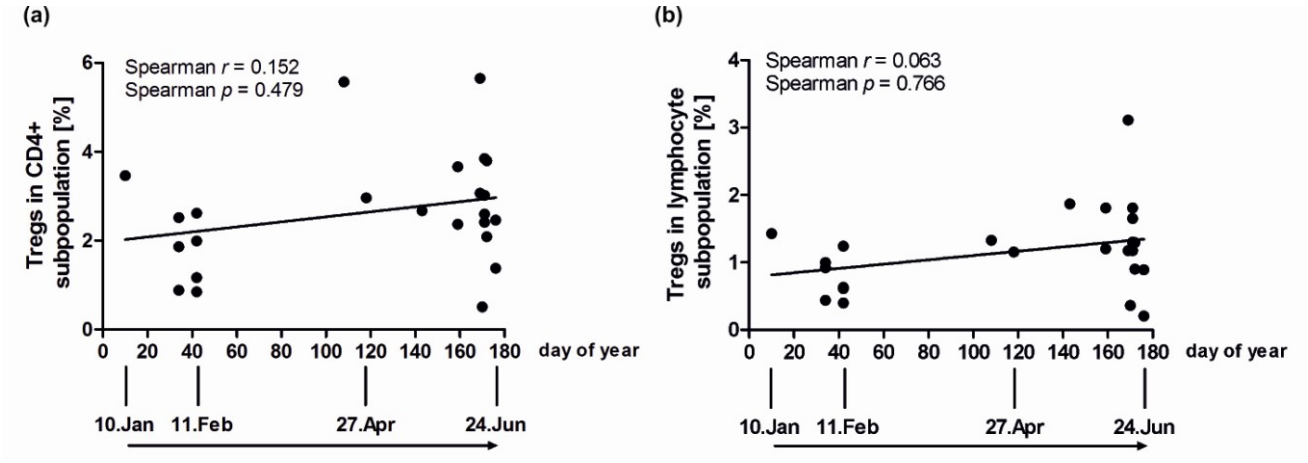

Figure 3.4.2. Tregs of the CD4+-or lymphocyte subpopulation show a seasonal tendency for an increase towards summer. PBMCs of PLE patients were stained with antibodies for CD4, CD127, CD25 and FoxP3. (a) depicts the individual percentages of Tregs as a proportion of CD4+ cells and (b) represents the individual percentages of Tregs in all lymphocytes plotted

against day of year. (a) and (b) n = 24 PLE patients; (a) and (b) *r*- and *p*-values were determined using Spearman correlation.

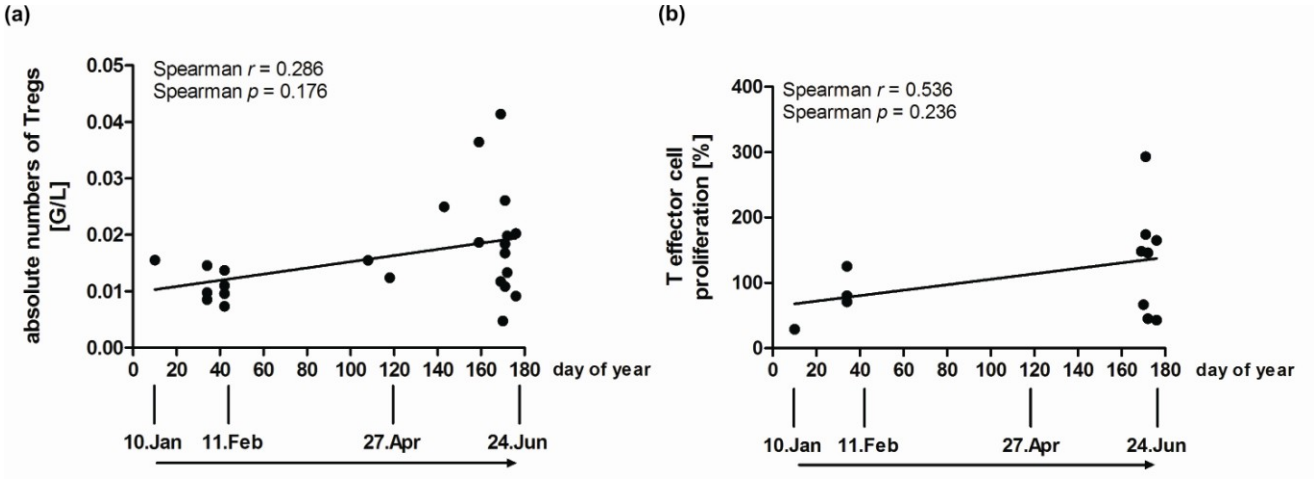

Figure 3.4.3. Absolute Tregs also show a seasonal tendency in increase of numbers towards summer but no increase in function (i.e. more suppression of Teff cell proliferation). (a) Individual absolute Treg numbers from PLE patients were determined at TP1 by flow cytometry and calculated using leukocyte counts by automated blood cell analysis. (b) Individual Treg suppression assays from PLE patients plotted against day of year. Data shown are individual 1:1 ratios, normalised to T effector cell proliferation alone (0:1 ratio = 100%). 1:1 ratio represents the results of co-culture of equal numbers of T effector (CD4+CD25-CD127+) and Tregs (CD4+CD25+CD127-). (a) n = 24 PLE patients, (b) n = 12 PLE patients. (a) and (b) *r*- and *p*-values were determined using Spearman correlation.

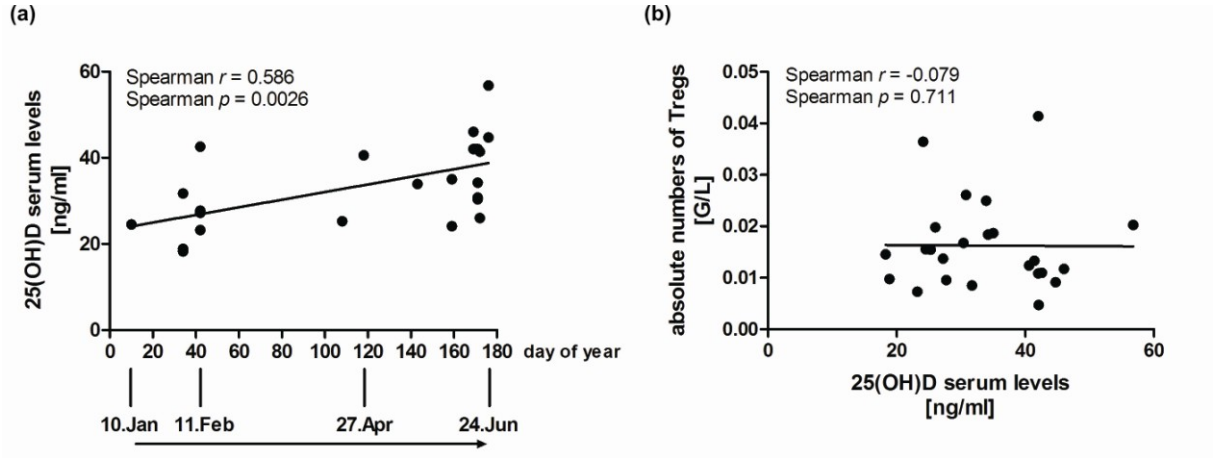

Figure 3.4.4. 25(OH)D serum levels statistically correlate with day of year but not with absolute Treg numbers in PLE patients. (a) Blood samples from PLE patients were collected at TP1 and individual 25(OH)D serum levels were determined and plotted versus day of year. (b) Absolute numbers of Tregs from PLE patients are plotted against their respective 25(OH)D serum levels. (a) and (b) n = 24 PLE patients. *r*- and *p*-values were determined using Spearman correlation.

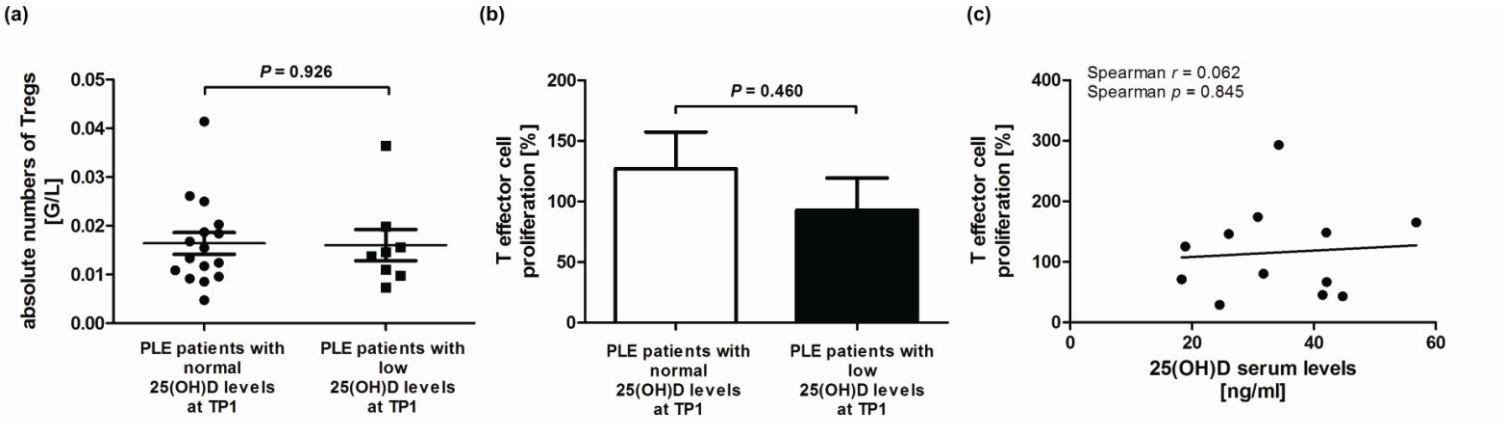

Figure 3.4.5. Treg levels and function are similar in PLE patients with normal or low 25(OH)D serum levels at TP1. (a) Absolute numbers of Tregs from PLE patients with low and normal 25(OH)D serum levels measured at TP1 are shown. (b) Treg suppression assays from PLE patients at TP1 plotted against day of year. Data shown are individual 1:1 ratios, normalised to T effector cell proliferation alone (0:1 ratio = 100%). 1:1 ratio represents the results of co-culture of equal numbers of T effector (CD4+CD25-CD127+) and Tregs (CD4+CD25+CD127-). (c) Individual proliferation assays of patients plotted against their respective 25(OH)D serum levels. (a) n = 16 PLE patients with normal- and 8 PLE patients with low 25(OH)D serum levels; (b) n = 8 PLE patients with normal and 4 PLE patients with low

25(OH)D serum levels; (c) 12 PLE patients; *P*-values were determined by Mann-Whitney test.

3.5 TABLES

Table 3.5.1 Characteristics of PLE patients.

Characteristics	Patients with normal 25(OH)D serum levels	Patients with low 25(OH)D serum levels
Gender	f: 12; m: 4	f: 7; m: 1
Age in years (range)	45.7 (25-68)	47.6 (25-76)
Skin phototype*, n	I: 1; II: 5; III: 9; IV: 1	II: 2; III: 6
Mean disease duration in years (range)	22.5 (1-42)	13.5 (1-29)
Establishment of diagnosis	**PGPH: 15; CE: 1; P: 1; H: 0	PGPH: 8; CE: 2; P: 2; H: 2
Results of photoprovocation (years before study start and at study start)	*** UVA/UVB: 1; SSR: 0; neg: 2; nd: 13	UVA/UVB: 1; SSR: 1; neg: 1; nd: 5
Pathology of PLE lesions	**** mp: 3; pap: 6; pv: 6; v: 1	mp: 2; pap: 5; pv: 1; v: 0
Predilection body site	V-neck: 13; arm: 10; forearm: 8; thighs: 4; abd.: 3; whole body:1; back:3; feet:0; face:2	V-neck: 8; arm: 3; forearm: 6; thighs: 2; abd.:0; whole body:0; back:0; feet:2; face:0
25(OH)D serum levels at TP1 (mean with range)	38.7 (25.3-56.8)	23.7 (18.3-27.7)

* According to Fitzpatrick classification; ** PGPH = Physician-guided patient history, as described in Patients and Methods; CE = Clinical examination at presentation of the disease; P = Photoprovocation; H = Histology; *** UVA/UVB = UVA positive; UVB = UVB positive; SSR = solar simulated radiation positive; neg = no skin reaction after laboratory photoprovocation with near-erythematogenic daily exposure over up to 4 days with UVA or UVB; nd = not done; **** mp = maculopapular; pap = papular; pv = papulovesicular; v = vesicular; abd. = abdomen

Table 3.5.2. Time table of the original study

Study day (time point)	**Actions**
1 (TP1)	Blood sample collection for 25(OH)D determination, 1st MED testing
2 - 5	1st Photoprovocation testing
8	1st Photoprovocation reading, 1st administration of vitamin D or placebo
22 (TP2)	Blood sample collection, 2nd administration of vitamin D or placebo
36 (TP3)	Blood sample collection (TP3), 2nd MED testing, start of 2nd photoprovocation
43	2nd Photoprovocation reading, handing out of questionnaire

4. STUDY PART 3: ROLE OF MAST CELLS IN PHOTOTOLERANCE INDUCTION AND PHOTO ITCH

Aims

Dermal mast cells play a key role in UVB-induced immune suppression, where they transmit the suppressive signal generated in mouse skin to the immune system by UV-induced migration of dermal mast cells to draining lymph nodes (Byrne et al., 2008). PLE patients were found to display decreased levels of papillary dermal mast cells that were restored upon medical photohardening therapy (Wolf et al., 2014). The exact immunological mechanisms behind photohardening remain to be determined. The aim of this thesis part was to investigate whether mast cells exert a potential mechanistic role in phototolerance through photohardening and to address the question: What happens in case of missing mast cells upon chronic or single UVB irradiation?

Parts of **"Role of mast cells in phototolerance induction and photo itch"** that are shown in the dissertation were published in Experimental Dermatology.

Nina A. Schweintzger, Isabella Bambach, Eleonora Reginato, Gerlinde Mayer, Alberto Y. Limón-Flores, Stephen E. Ullrich, Scott N. Byrne, and Peter Wolf

Mast cells are required for phototolerance induction and scratching abatement

Experimental Dermatology 2015, DOI: 10.1111/exd.12687

The permission for reprinting the content (text and figures) of the published manuscript for incorporation in my PhD thesis was granted by Thomas Trier-Mork, Journal Publishing Manager, Wiley-Blackwell.

4.1 MATERIALS AND METHODS

4.1.1 Animals

Kit$^{W\text{-}sh}$/HNihrJaeBsmJ transgenic mice (on C57Bl/6J background) expressing the c-*kit* mutation that renders them deficient in mast cells were obtained from the Jackson Laboratories, Bar Harbor, Maine. C57Bl/6J mice were used as controls and purchased from Charles River Laboratories, Sulzfeld, Germany. Animals were housed as described previously (Singh et al., 2010). Animal procedures were approved by the Austrian government, Federal Ministry for Science and Research, through protocol number BMWF-66.010/0086- II /3b/2011, and by the University of Texas MD Anderson Cancer Center's Animal Care and Use Committee. Mice were 9-13 weeks old at start of experiments and were sex- and age-matched.

4.1.2 UV irradiation

Dorsal hairs of mice were removed with an electric clipper 24h prior to UV exposure. UV radiation was performed using a Waldmann 236 light source (Waldmann Medizintechnik, Villingen-Schwenningen, Germany), equipped with two Waldmann UV6 fluorescent tubes (emission range 280-360 nm; peak, 320 nm); positioned upside down on top of cage (Pflegerl et al., 2009; Singh et al., 2014). This light source emitted a spectrum of approximate 40% UVB and 60% UVA radiation (Fig.4.4.1a); 99% of the erythema-producing radiation of this light source derived from the UVB region (280-320 nm) (Fig.4.4.1b), as determined by spectral measurement (MSS 2040 UV spectral radiometer, Fröndenberg, Germany) and calculation of the CIE-weighted erythema spectrum. The mean UVB irradiance of the lamp was 1.9 mW/cm^2, as measured by a Waldmann UV photometer with a UV6 detector head appropriate to the radiation device. The irradiation regimes consisted of three parts: part one was the estimation of the minimal skin swelling dose (MSSD, minimum dosage of UVB required to produce a skin swelling after 24h). This part is also called pre hardening MSSD regime in this work. Part two consisted of the photohardening regime and part three of the post hardening MSSD determination (for tabular and schematic representation see Table 4.5.1 and Figure 4.4.3). To estimate the pre hardening MSSD, 4 mice per group were irradiated with 30mJ/cm^2 on day 1 (based on previous studies (Wolf et al., 1993) with 40% increments in daily UVB dosage, until they showed a significant skin swelling response. Six days later, the photohardening regime was started with $MSSD_{50}$ (half of the MSSD determined for each of the

mouse groups). UV exposure was given twice a week for 4 weeks, with 20% increments in UVB dosage at each irradiation step. Four days after photohardening treatment ended, the animals were rechallenged with the post hardening UV regime.

4.1.3 Preparation and adoptive transfer of BMCMCs into mast cell-deficient mice

Bone marrow cell preparation and transplantation from 4 week-old female C57Bl/6J was performed as described (Kalesnikoff and Galli, 2011). Bone marrow cultured mast cells (BMCMCs) were stained before reconstitution with Naphthol (LEUCOGNOST® NASDCL, Merck, Darmstadt, Germany) and Giemsa. The purity was found to be > 98%. For mast cell reconstitution studies, 4×10^6 BMCMCs dispersed in 400 µl were injected intradermally in eight sites (with 50 µl aliquots per site) in two rows (4 injection sites per row) down the length of shaved back into 4-week-old female $Kit^{W\text{-}Sh/W\text{-}Sh}$ mice. The distance between each injection site was approximately 0,5cm. Eight weeks after intradermal transfer, reconstitution was confirmed by counting the number of Giemsa-stained mast cells in dorsal skin biopsies and UV irradiation experiments were started (Fig.4.4.2).

4.1.4 Assessment of skin scratching

Mice were observed immediately after each UV exposure for 30 min during the different regimes and each scratching attack was recorded. The total number of scratching bouts per 30 min per is given in the graphical presentations.

4.1.5 Assessment of skin thickness

Skin thickness was quantified by measuring double dorsal skin-fold thickness using a spindle-loaded pocket-sized thickness gage (Mitutoyo, Japan, model 7309) (Wolf et al., 1993). The mean skin thickness of the experimental groups was determined from three independent measurements of each mouse per day. Skin swelling was calculated by subtracting the skin thickness at day 0 from the skin thickness of a respective day (Fig.4.4.4a and b).

4.1.6 Tissue collection

Mice were sacrificed 24 hours after final treatment, at which point skin samples were collected and either immediately processed or snap-frozen and stored in liquid nitrogen or immersed in formaldehyde for further analysis.

4.1.7 Histology

3-µm thick paraffin-embedded sections of mouse dorsal or ear skin were stained with hematoxylin and eosin (H&E). Histologic evaluation of skin thickness was performed using an Olympus BX51 microscope acquired with a DP71 digital camera (Olympus, Vienna, Austria) choosing five randomly selected areas in the epidermis, dermis and/or subcutis with a magnification of 20x. Thickness and blood vessel area was calculated using cellSens standard image software (Olympus solutions, Münster, Germany). An ocular grid with area coverage of 0.25mm^2 was used for blood vessel area and mast cell number detection. For neutrophil infiltration cells in hot spots were counted and an ocular grid of 0.06mm^2 was used. For graphical presentation cell numbers were calculated per mm^2.

4.1.8 Immunohistochemistry and immunofluorescence

Treg cell immunofluorescence staining was performed on paraffin-embedded sections of mouse dorsal skin with eFluor 615-conjugated anti-mouse/rat FoxP3 (1/20) (eBioscience, Vienna, Austria) and polyclonal rabbit anti-mouse CD4 (1/50) (Clone A429; Novus Biologicals, Littleton, Colorado). Alexa-Fluor 488 conjugated goat anti-rabbit IgG (H+L; Invitrogen, Molecular Probes, Eugene, Oregon) (1/200) was used as secondary antibody. Neutrophils were stained with rat-monoclonal anti-mouse neutrophil antibody [NIMP-R14] (1/50) (Abcam, Cambridge, Massachusetts). Biotinylated anti-rat IgG (H+L; Vector Laboratories, Burlingame, California) was used as secondary antibody (1/100) and visualized with DakoREAL Streptavidin Peroxidase (HRP) (Dako, Vienna, Austria). Sections were immersed in EDTA (pH 8) and put in a vegetable steamer for 30 min. We used Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, California) for immunofluorescence stainings. Giemsa staining (Merck Millipore, Darmstadt, Germany) was used to compare mast cell numbers in dorsal skin of mice. Images of stainings were acquired with a DP71 digital camera (Olympus), attached to an Olympus BX51 microscope.

4.1.9 Statistical analysis

Scorings of microscopic slides and macroscopic readings were conducted in a blinded manner. Data presented are expressed as means ± SD and means ± SEM. Statistical differences were determined by using Kruskal-Wallis or Friedman test with Dunn's correction. All analyses were performed with Prism 5.0; statistical significance was set at $P< 0.05$.

4.2 RESULTS

4.2.1 Mast cells are critical for reducing susceptibility to UV-induced inflammation by inducing phototolerance through photohardening treatment

Before starting the photohardening treatment, the mean minimal skin swelling dose (MSSD) for each mouse group was determined through a prehardening regime (see Table 4.5.1 for a detailed description of the UV regimes). C57Bl/6J mice, serving as the control group, showed significant dorsal skin swelling on day 8, 24h after receiving 316 mJ/cm^2 of UV radiation (Fig.4.4.3a for schematic representation and Fig.4.4.4a). This is consistent with what has previously be found for this strain (Byrne et al., 2002). Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice and their mast cell-reconstituted counterparts exhibited a slightly lower mean MSSD (225 mJ/cm^2) (Fig.4.4.4a; Table 4.5.2). Similar results were obtained for the ear swelling response (data not shown). The role of mast cells in phototolerance (i.e. decreasing the sensitivity to UV radiation) were determined through photohardening therapy (i.e. a prophylactic treatment), using a chronic UV irradiation regime that consisted of two sub inflammatory UV exposures per week given over 4 weeks, starting at a dose of 50% of the MSSD ($MSSD_{50}$) for the different treatment groups (Fig.4.4.3b). The protocol and light source (with its erythema-causing irradiation mainly coming from the UVB waveband) that was used were similar to that administered for prophylactic photohardening in photodermatoses in humans (Gruber-Wackernagel et al., 2014). The C57Bl/6J and mast cell-reconstituted Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice showed no swelling of dorsal skin during the course of the photohardening regime up to the 1.8-fold MSSD (564 and 406 mJ/cm^2, respectively) (Fig.4.4.5a). This was in contrast to the Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice, who demonstrated a noticeable skin swelling of dorsal skin at 1.2 MSSD (282 mJ/cm^2). Similar relative differences were obtained for the UV-induced ear swelling response (Fig.4.4.5b), where the ear skin of Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice displayed a significant increase in thickness at 406 mJ/cm^2, compared to BMCMC-reconstituted mice or WT mice. Four days after photohardening treatment it was investigated with a post hardening UV regime whether the mice were now able to tolerate higher UVB dosages (for schematic representation see Fig. 4.4.3c). Therefore they were subjected to the same irradiation regime used for pre hardening MSSD determination. After photohardening C57Bl/6J mice tolerated a UV dose that was 40% higher than their initial MSSD (442 vs. 316 mJ/cm^2) (Fig.4.4.4a and b; Table 4.5.2). Photohardening was particularly successful in mast cell-engrafted Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice, which could sustain almost a doubling in their respective MSSD (442 vs. 225 mJ/cm^2) before showing

signs of skin swelling and inflammation (Fig.4.4.4b and a; Table 4.5.2). In contrast, the skin of $Kit^{W\text{-}Sh/W\text{-}Sh}$ mice exhibited significant skin swelling at exactly the same UVB dose (225 mJ/cm^2) as before therapy (Fig.4.4.4b and a; Table 4.5.2). Thus, photohardening leading to more UVB tolerance was dependent upon the presence of mast cells.

4.2.2 Mast cells protect from photo itch

During determination of the pre hardening MSSD, an intense scratching behaviour of the $Kit^{W\text{-}Sh/W\text{-}Sh}$ mice immediately following UV exposure was observed (Fig.4.4.4c). Scratching was induced in these mice by doses of UVB that were only a quarter of that required to cause edema (i.e. 59 mJ/cm^2 vs 225mJ/cm^2) (Fig.4.4.4a and c; Table 4.5.2). Furthermore, bouts of scratching increased with longer UV exposures in a dose dependent manner (Fig.4.4.4c). UV-induced scratching in $Kit^{W\text{-}Sh/W\text{-}Sh}$ mice was mainly directed to the lower back and neck areas. Mice also displayed excoriations behind their ears and some of them exhibited signs of scratching on the abdomen and throat areas (data not shown). The UV-induced scratching was dependent on mast cells because $Kit^{W\text{-}Sh/W\text{-}Sh}$ reconstituted with WT mast cells lost this phenotype and became similar to that of WT mice (Fig.4.4.4c). An exacerbation of the scratching behaviour of $Kit^{W\text{-}Sh/W\text{-}Sh}$ mice was also observed during the post hardening MSSD regime (Fig.4.4.4d). Irradiation with as little as 42 mJ/cm^2 was now able to induce significant bouts of scratching, which again increased with UV dose. As before, UV did not induce significant scratching in either C57BL/6J or $Kit^{W\text{-}Sh/W\text{-}Sh}$ engrafted with BMCMCs (Fig.4.4.4d). The MSSD and minimal photo-itch dose (MPID) of the different groups of mice are summarised in Table 4.5.2.

4.2.3 The absence of mast cells renders UV-exposed mice susceptible to excessive cutaneous blood vessel dilation, increased edema and epidermal hyperplasia

Naive (unirradiated) mice of the three groups were exposed to 1 MSSD (see Fig.4.4.4a) in order to deliver a single dose of UVB. Skin samples were collected 0h, 24h and 72h after irradiation. The double skin-fold thickness was measured before collecting each sample. Compared to the BMCMC-reconstituted $Kit^{W\text{-}Sh/W\text{-}Sh}$ and WT control groups, the skin of $Kit^{W\text{-}Sh/W\text{-}Sh}$ mice was found to be significantly thicker 24h and 72h after irradiation (Fig.4.4.6a). Histologic evaluation of H&E-stained sections confirmed an increased epidermal thickness 24h and 72h after irradiation within each mouse group compared to 0h (Fig.4.4.6b). However, the

increase in epidermal thickness observed in Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice 72h after irradiation was significantly higher than in the other groups. Skin biopsies of photohardened mice taken 24h after rechallenge MSSD exposure (Fig.4.4.4b) also showed an extensive increase in epidermal thickness but there was no significant differences between the groups (Fig.4.4.6b). There was also no significant difference between any group at any time point when comparing the number of epidermal cell layers (Fig.4.4.6c). The dermis in H&E stained sections was measured and found to be thicker 24h and 72h after UV in naive mice as well as 24h after rechallenge MSSD in mast cell-deficient photohardened mice (Fig.4.4.6d) compared to both control groups. No difference in subcutis thickness was observed among the groups and time points investigated (data not shown). Naive Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice displayed an increased dermal blood vessel area 24h and 72h after UV exposure compared to baseline as well as WT and BMCMCs-reconstituted mice (Fig.4.4.6e and h). The blood vessel area of rechallenged Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice was also significantly increased compared to the control groups (Fig.4.4.6e). Mast cell-sufficient groups also showed an increase of that area at the same time points but this was not as prominent. All three groups of previously unirradiated (naive) mice showed a significant increase in subcutis blood vessel area 72h after their first irradiation (Fig.4.4.6f). This increase also occurred in photohardened mice 24h after rechallenge MSSD exposure (Fig.4.4.6f). The absolute number of blood vessels in the dermis was counted and a significant increase was found 24h after the last exposure of rechallenge MSSD in all groups of photohardened mice (Fig.4.4.6g). In contrast to blood vessel area (Fig.4.4.6f), the number of blood vessels in the subcutis showed no significant difference among any group at any time point investigated (data not shown).

4.2.4 A lack of mast cells is linked to a failure to recruit Tregs and neutrophils into UV-irradiated skin

Upon UV irradiation the skin is infiltrated by neutrophils which together with the participation of other immune cells culminates in the activation of Tregs, suppressing inflammation and adaptive immunity (Curotto de Lafaille and Lafaille, 2009; Maeda et al., 2008; Schwarz and Schwarz, 2010; Schwarz et al., 2011; Umetsu et al., 2003). The influence of missing mast cells on UV-irradiated mouse skin and its impact on the cascade of immunosuppressive events was therefore investigated. The number of $CD4^+FoxP3^+$ Tregs in dorsal skin sections from naive mice sacrificed 0h, 24h and 72h after exposure to 1 MSSD as well as from photohardened mice 24h after the final rechallenge MSSD exposure was determined. Compared to WT con-

trols there was a significantly reduced number of Tregs both in the unirradiated and 24h post UV dorsal skin of Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice (Fig.4.4.7a). This defect was restored in mast cell-reconstituted Kit$^{W\text{-}Sh/W\text{-}Sh}$. A difference in Treg numbers in blood, lymph nodes or spleen among the different groups of mice by flow cytometry could not be observed (data not shown). When skin sections were stained for neutrophils it was found that Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice had lower numbers of neutrophils at baseline (0h) compared to WT controls (Fig.4.4.7b). Moreover, a defect in the capacity of Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice to recruit neutrophils to sites of UV exposure was observed (compared to C57Bl/6J mice). These lower neutrophil numbers at baseline and 24h following UVB exposure were dependent on mast cells, because the defect was corrected in mast cell reconstituted Kit$^{W\text{-}Sh/W\text{-}Sh}$ (Fig.4.4.7b). Interestingly, by 72h post UV the numbers of cutaneous neutrophils did not statistically differ among the naive groups. Moreover, a significantly decreased neutrophil infiltration into the skin of un-successfully photohardened Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice compared to successfully photohardened WT mice was observed. Photohardened BMCMCs-reconstituted control mice displayed similar neutrophil numbers to C57Bl/6J mice (Fig.4.4.7b).

4.3 DISCUSSION

Using Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice, evidence for the requirement of mast cells in the development of phototolerance is provided. WT mice and Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice engrafted with mast cells could withstand higher doses of UVB exposures without exhibiting skin swelling following photohardening therapy (Fig.4.4.4b and Table 4.5.2). This did not occur in Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice that remained significantly more susceptible to UVB-induced skin swelling. The increased skin swelling in mast cell-deficient mice upon single and chronic UV exposure was mainly due to an increased thickness of the dermis, presumably caused by edema that resulted from leakage of fluid from enlarged blood vessels (Fig.4.4.6e and h). Although an increase in dermal blood vessel number of photohardened mice 24h after exposure to the final rechallenge MSSD was observed, this did not appear to be dependent on mast cells as it occurred in all groups with no significant differences between them (Fig.4.4.6g). Therefore the mechanism for the enlargement of dermal blood vessels following UV exposure of Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice remains unknown at present. The observation of increased skin edema in Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice exposed to UV is consistent with what has previously been described by Grimbaldeston *et al.* (Grimbaldeston et al., 2007) using mast cell-deficient Kit$^{W/W\text{-}v}$ mice. They showed that mast cell-derived IL-10 was required to prevent inflammatory leukocyte infiltration and limit the inflammation induced by chronic low dose UVB irradiation. In contrast, the photohardened Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice in this part of the thesis exhibited decreased neutrophil numbers in the skin 24h after last exposure of rechallenge MSSD, compared to WT controls and mast cell-engrafted Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice (Fig.4.4.7b).

Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice were used, which have fewer developmental and phenotypic abnormalities than Kit$^{W/W\text{-}v}$ mice to investigate the effect of defined UV doses. It is known, that the *Kit*$^{W\text{-}v}$ mutation which is directly located at the tyrosine kinase domain of *c-kit* leads to sterile, anemic mice with a marked deficiency of intraepithelial lymphocytes in the small intestine (Grimbaldeston et al., 2005; Nocka et al., 1990; Puddington et al., 1994). These mice also display severe neutropenia (Zhou et al., 2007), lack basophils (Mancardi et al., 2011) and develop spontaneous stomach papillomas and ulcers as well as idiopathic dermatitis (Galli et al., 1987; Kitamura et al., 1980; Shimada et al., 1980). In contrast, mice harbouring a *Kit*$^{W\text{-}sh}$ mutation (an inversion in regulatory elements upstream of the c-kit element) lack all of the abnormalities mentioned above (Nagle et al., 1995). In both mice strains the mutations result in

an abolishment of mast cells, melanocytes and interstitial cells of Cajal in the gut, soon after birth (Besmer et al., 1993; Duttlinger et al., 1993; Grimbaldeston et al., 2005).

Evidence was provided that mast cells play a key role in induction of skin swelling phototolerance through photohardening treatment, as in contrast to $Kit^{W\text{-}Sh/W\text{-}Sh}$ mice, mast cell-reconstituted littermates displayed the same swelling pattern as the WT C57Bl/6J controls. It should be noted that a slight "hardening effect" in $Kit^{W\text{-}Sh/W\text{-}Sh}$ was observed, as these mice did display a less pronounced skin swelling during the post hardening MSSD regime at a dose of 225 mJ/cm^2 (i.e. approximately 30 vs. 15 x 10^{-2} mm comparing pre vs. post hardening regime) (Fig.4.4.4a and b). One possible explanation for this is UVB-induced epidermal hyperplasia (Fig.4.4.6b and c) that mast cell-independently may have led to photo adaption. The Grimbaldeston et al. study (Grimbaldeston et al., 2007) and this part of the thesis would appear to conflict with other reports of mast cells actually being required for UV-induced inflammation (Ikai et al., 1985; Metz et al., 2006a). The most likely explanation for the differences is that while our mice were exposed to a twice-weekly regimen of chronic UV, these earlier studies used a single, acute UVB exposure.

Surprisingly, the increased skin swelling in the mast cell-deficient mice upon UV exposure was partially independent from skin pigmentation, as during the photohardening regime and at the post hardening MSSD regime (but not in the pre hardening MSSD regime) the (non-pigmented) mast cell-reconstituted $Kit^{W\text{-}Sh/W\text{-}Sh}$ mice exhibited an equivalent amount of skin swelling than the pigmented WT mice at similar UVB doses (Fig.4.4.4b and Fig.4.4.5a and b). It is known, that by injecting BMCMCs in dorsal back or ear pinna of mast cell-deficient mice, a local reconstitution can be achieved (Grimbaldeston et al., 2005; Wolters et al., 2005). However, in $Kit^{W\text{-}Sh/W\text{-}Sh}$ mice reconstituted with mast cells into the dorsum only and undergoing our photohardening regime a reduction in both dorsal-skin and ear-skin swelling to levels that were similar to WT mice was observed. Mast cells injected into dorsal back skin may have migrated to the ears via the circulation. Indeed, a few mast cells in local skin-draining lymph nodes of reconstituted mice after photohardening were found (data not shown).

Itch is a major symptom of certain photodermatoses, including polymorphous light eruption (PLE) (Gruber-Wackernagel et al., 2011; Gruber-Wackernagel et al., 2014b) and solar urticaria (Beattie et al., 2004, 2003; Du-Thanh et al., 2013). This hitherto unrecognized role for mast cells in protecting from, rather than causing itch, conflicts with conventional wisdom that mast cells initiate itching during type 1 allergic reactions. It was found that a deficiency

in mast cells was responsible for this phenotype by showing that engraftment of WT mast cells into Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice led to an ablation of their UV-induced scratching behavior (Fig.4.4.4c and d). Previous photocarcinogenesis studies in hairless SKH:HR1 mice (de Laat et al., 1997) and Fabpl*Cre*;*Apc*$^{15lox/+}$ mice (Rebel et al., 2015) showed that chronic UVA radiation also lead to a scratching phenotype. Interestingly, mast cell-release of histamine and/or serotonin was not responsible for this phenotype, nor did UVA affect mast cell numbers (de Laat et al., 1997). In this part of the thesis the use of an Oriel solar simulator which reproduces the entire UV spectrum of sunlight or UV6 lamps emitting a spectrum of approximate 40% UVB and 60% UVA radiation (Fig.4.4.1a) both induced photo-itch (Fig.4.4.4c and d). It is therefore not entirely clear whether UVA or UVB wavebands (or both) are responsible for UV-induced pruritus. It furthermore remains to be determined how mast cells protect from photo-itch and scratching. One possibility is a failure to clear UV-induced endothelin-1 (ET-1) (Tsuboi et al., 1995) which can cause a short-lived burning itch in humans (Katugampola et al., 2000) and mice (Trentin et al., 2006). ET-1 can be produced and bound by a number of cutaneous cells including keratinocytes, endothelial cells and mast cells (Ehrenreich et al., 1992; Tsuboi et al., 1995; Yanagisawa et al., 1988). Binding of ET-1 to ET_A receptors on the surface of mast cells induces their degranulation. The subsequent cascade of inflammatory mediator release, amplifies the developing inflammatory response (Szalay et al., 2000; Yamamura et al., 1994). However, ET-1 binding to ET_A receptors on mast cells also triggers the release of carboxypeptidase A and possibly other proteases that subsequently breakdown ET-1. In this way, mast cells are responsible for reducing high ET-1 levels and maintaining homeostasis (Maurer et al., 2004). In the absence of this homeostatic pathway, a build-up of non-degraded ET-1 would evoke itch by binding neuronal TRP ankyrin A1 (TRPA1) channels in mice (Liang et al., 2011) or stimulating itch-sensitive neurons in humans (Kido-Nakahara et al., 2014).

A reduced number of cutaneous mast cells, as was recently reported for PLE patients (Wolf et al., 2014) or a lack of them (like in Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice) could render a UV-exposed individual susceptible to rising ET-1 levels and precipitate excessive itching. This interpretation would be consistent with the prevailing view that in addition to their traditional role in mediating atopy, mast cells are also required to maintain homeostasis.

In acute bacterial infections mast cells and mast cell products promote neutrophil infiltration which helps clear bacterial manifestations (Echtenacher et al., 1996). In the *in vivo* studies it was observed that mast cells provided recruiting signals for neutrophils after UV irradiation,

since a decreased infiltration of neutrophils into the skin of Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice 24h after irradiation was found (Fig.4.4.7b). However, 72h after UV irradiation, the neutrophil infiltration in mast cell-deficient mice reached the same levels as found in the WT controls and reconstituted mice, possibly due to the influence of non-mast cell-linked stimuli. Another type of immune cells that was present in decreased numbers in the dorsal skin of our Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice 0h and 24h after irradiation were Tregs, paralleling the defective infiltration of neutrophils at the same time points. Mast cells or their products might play a role in the establishment and migration of Tregs into the skin after UV irradiation, since reconstituted Kit$^{W\text{-}Sh/W\text{-}Sh}$ displayed the same Treg levels as the WT control. This defect was observed only in skin and not in blood, lymph nodes and spleen. Any difference in Treg numbers among the photohardened mice 24h after last exposure of rechallenge MSSD could not be observed (Fig.4.4.7a), most likely due to the high UV dosages given towards the end of the regime (316 mJ/cm^2 and 442 mJ/cm^2; see Fig.4.4.4b).

In this part of the thesis it was discovered that Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice are prone to develop photoitch and are susceptible to UV-induced skin swelling. It was also found that they have a defect in the early recruitment of neutrophils to the skin after both single and chronic UV exposures and fail to induce Tregs. The resistance to UV-induced immune suppression in mast-cell deficient Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice is similar to that in patients with PLE (Janssens et al., 2005; Kölgen et al., 1999; Koulu et al., 2010; Palmer and Friedmann, 2004; van de Pas et al., 2004). This part of the thesis has thus identified mast cell-deficient Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice as a new *in vivo* model to investigate photodermatoses.

4.4 FIGURES

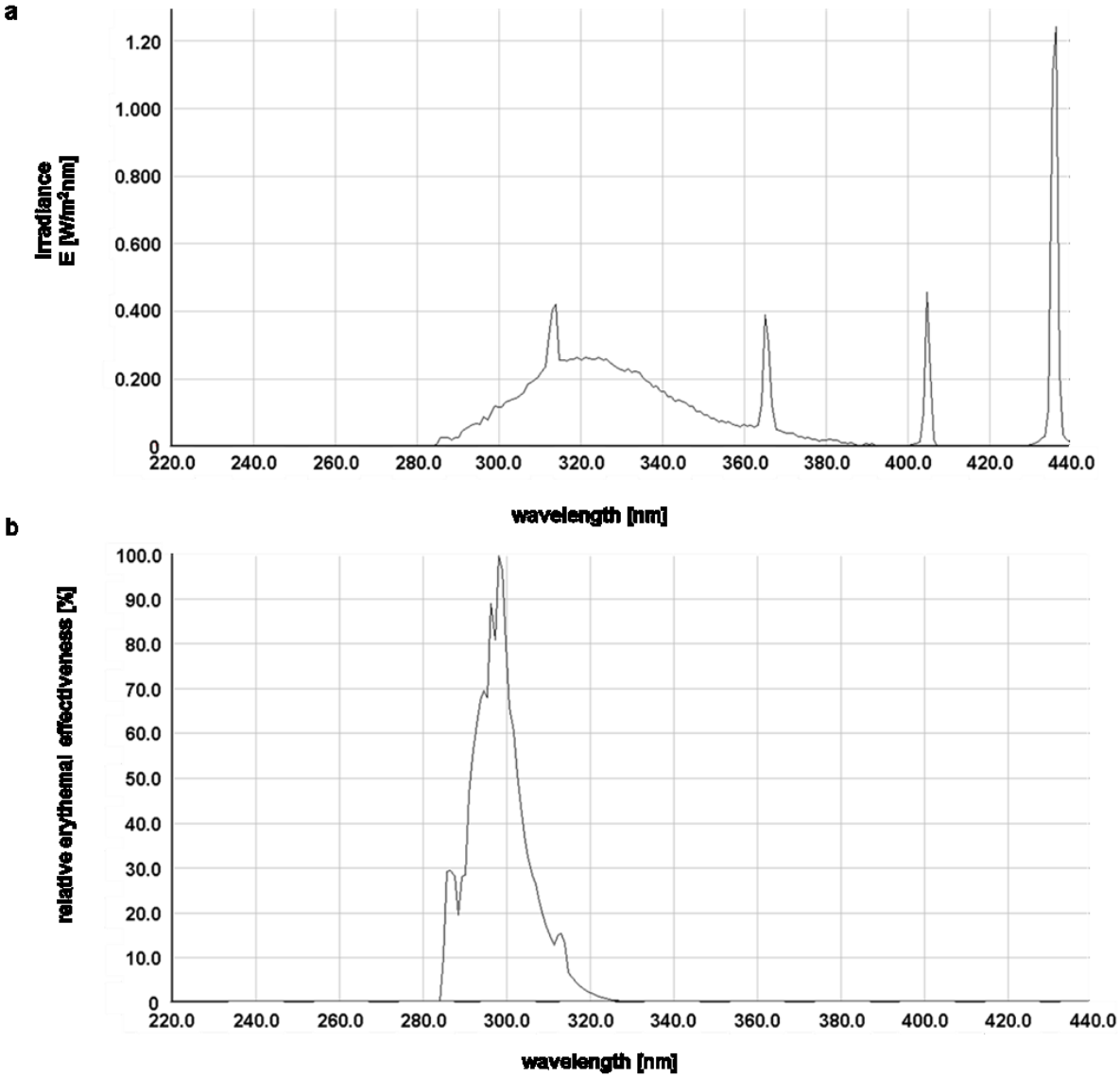

Figure 4.4.1. 99% of the erythema-producing radiation of the UV6 light source comes from the UVB region (280-320 nm). (a) The UV spectrum of the light source with its UV6 fluorescent tubes and (b) the erythema spectrum (erythema-producing radiation) were determined by spectral measurement with a spectral radiometer, as described in materials and methods.

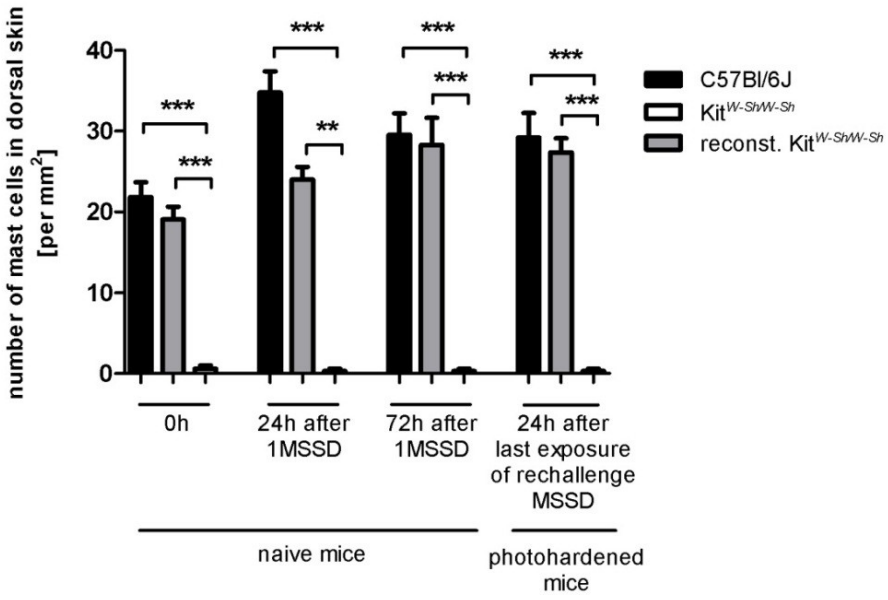

Figure 4.4.2. Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice are successfully reconstituted with BMCMCs from WT mice. Comparison of mast cell numbers in dorsal skin of C57Bl/6J, Kit$^{W\text{-}Sh/W\text{-}Sh}$, and reconstituted Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice at start of experiments, 24 and 72h after 1MSSD (naive, non photohardened mice) as well as 24h after last exposure of rechallenge MSSD (see Figure 4.4.4b). n = 4 mice per group; mean ± S.D. shown; $^{**}P < 0.01$; $^{***}P < 0.001$ (Kruskal-Wallis with Dunn's correction).

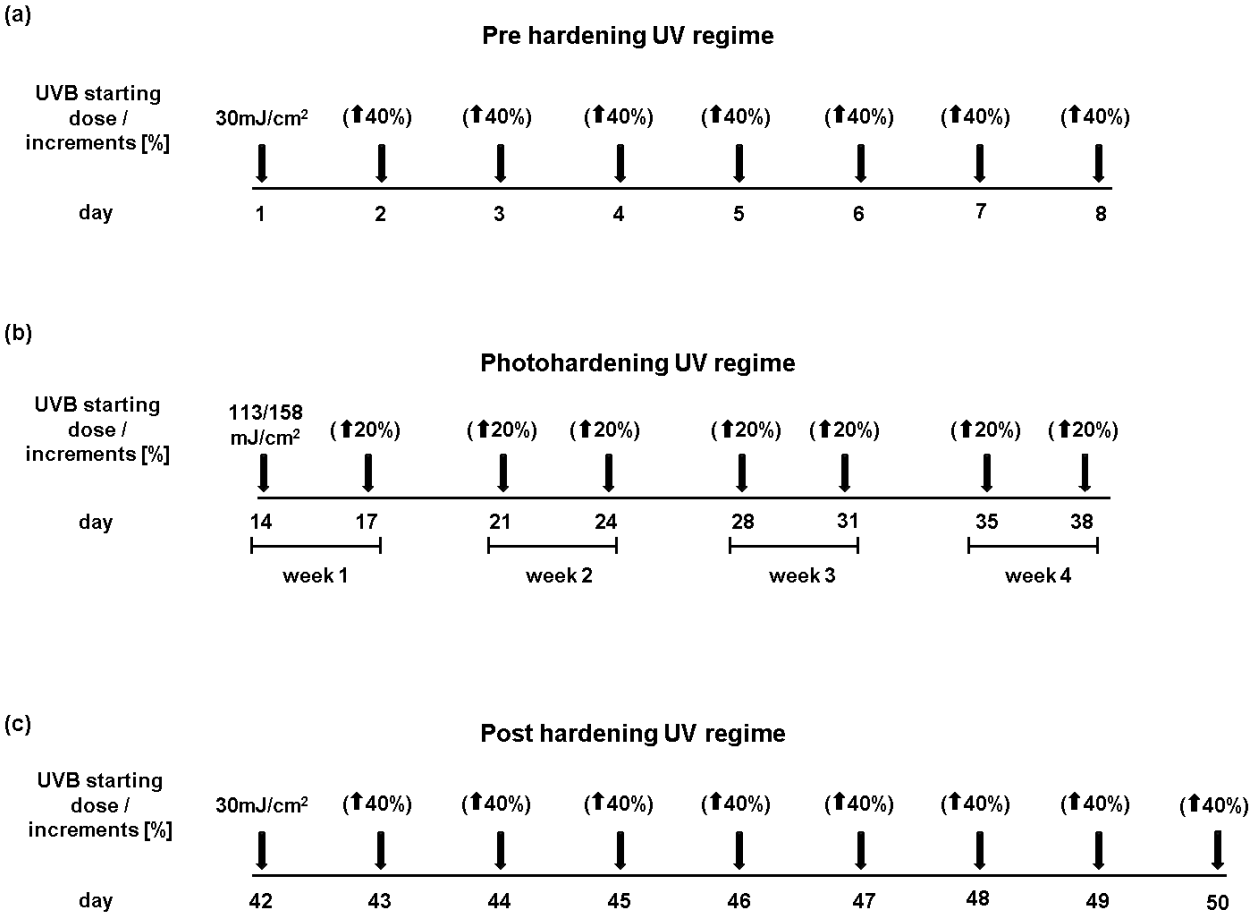

Figure 4.4.3. Schematic outline of the three irradiation regimes. Mice were irradiated with different UV regimes to determine their minimal skin swelling dose (MSSD) and relative minimal photo itch dose (MPID) in order to detect a change in sensitivity to subsequent UV irradiation after a photohardening treatment. (a) Pre hardening MSSD determination from day 1-8 with 40% increase in daily UVB dose. (b) Photohardening treatment was started at a dose of 113 mJ/cm^2 for Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice and their reconstituted counterparts and 158 mJ/cm^2 for C57Bl/6J mice. It was given twice a week for 4 weeks with 20% increments at each exposure. (c) Post hardening regime given from day 42-50 with 40% increments in UV dosage at each exposure.

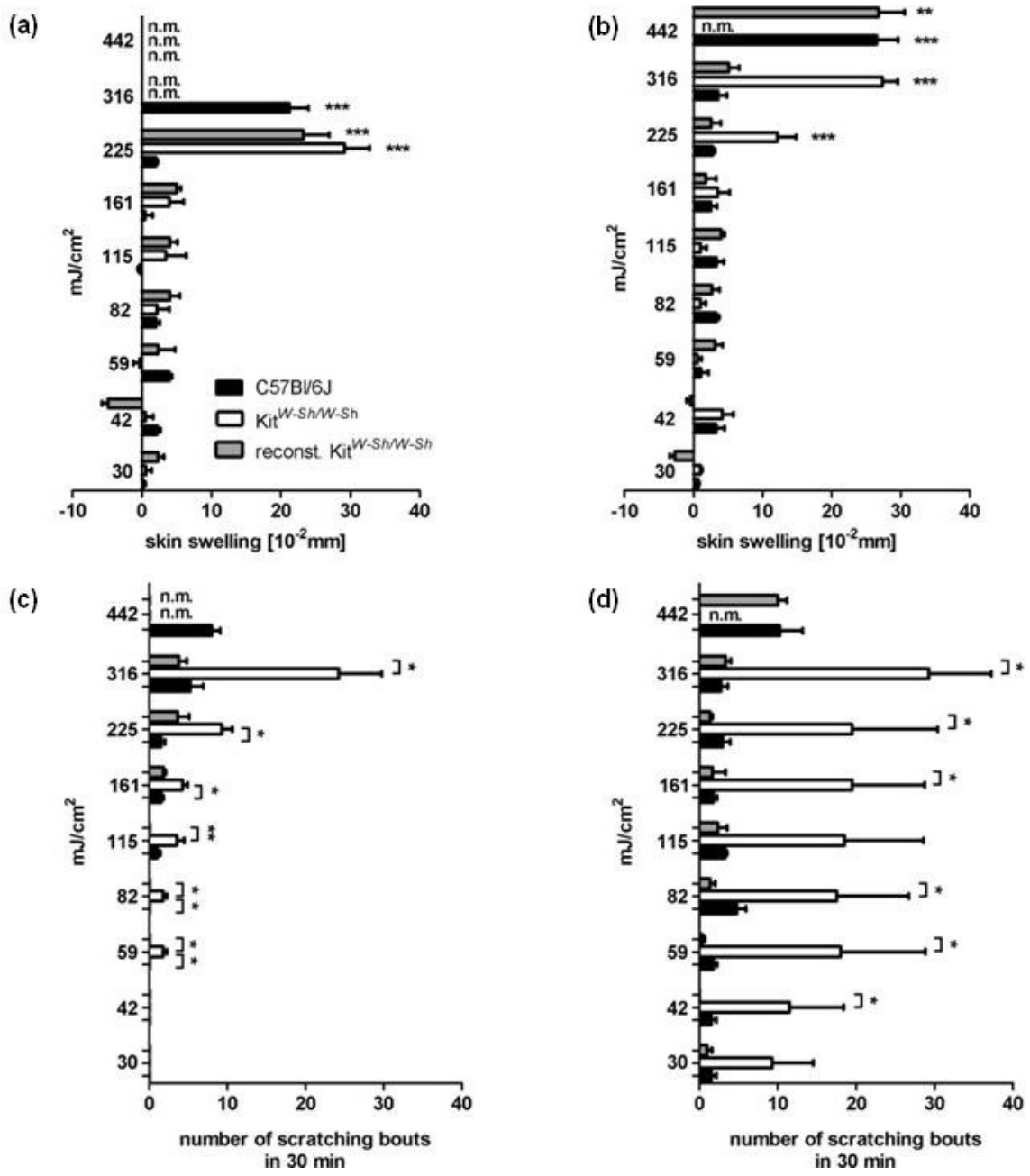

Figure 4.4.4. Mast cell-reconstitution in Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice abates UV-induced scratching. Mice were irradiated with 40% increments in UVB dose at each exposure before and after photo-hardening regime until they showed a significant skin swelling, at which point UV exposures were stopped. Skin swelling was measured 24h after each irradiation step during (a) pre hardening, and (b) post hardening UV regime. Scratching behaviour was monitored and bouts of scratching were recorded for 30 min after each UV exposure at (c) pre hardening, and (d) post hardening UV regime. (a-d), n = 4 mice per group. (a,b) = mean ± S.D.; (c,d) = mean ± S.E.M; n.m.; not measured; * P <0.05; ** $P \leq 0.01$; *** $P \leq 0.001$; (a,b) Friedman test with Dunn's correction was used to compare skin thickness within each mouse group at day 0 (be-

fore UV exposure) to each subsequent irradiation dose; (c,d) Kruskal-Wallis test with Dunn's correction.

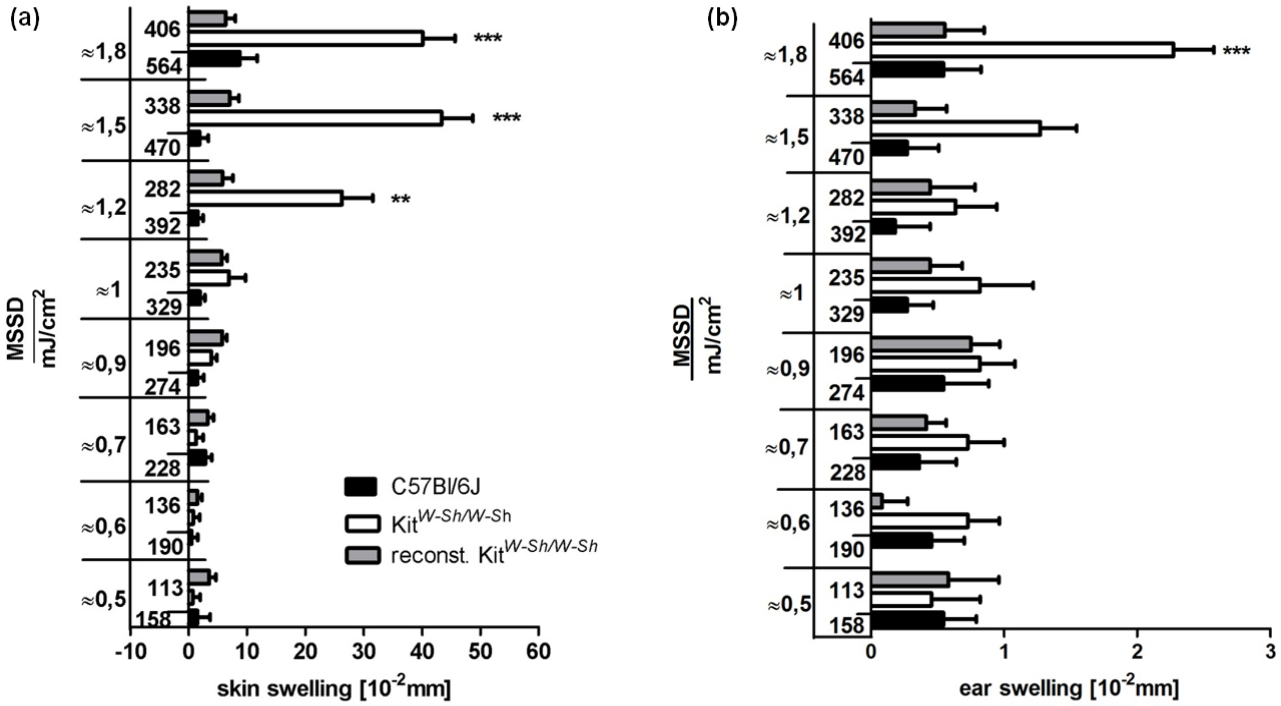

Figure 4.4.5. Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice display extensive dorsal skin and ear swelling during photohardening treatment. Mice underwent a photohardening treatment that was started at a dose of 50% of the MSSD ($MSSD_{50}$) for the different treatment groups as determined in Figure 4.4.4a (C57B/6J received 158mJ/cm^2; Kit$^{W\text{-}Sh/W\text{-}Sh}$ and reconstituted Kit$^{W\text{-}Sh/W\text{-}Sh}$ received 113mJ/cm^2). UV exposure was given twice a week for 4 weeks with 20% increments in UVB dosage at each irradiation step. (a) Dorsal skin and (b) ear swelling was measured 24h after each exposure. n = 4 mice per group; mean ± S.D. shown; mJ/cm^2 and their respective MSSD are shown; ** $P \leq 0.01$; *** $P \leq 0.001$; Friedman test with Dunn's correction was used to compare skin thickness within each mouse group at day 0 (before UV exposure) to each subsequent irradiation dose.

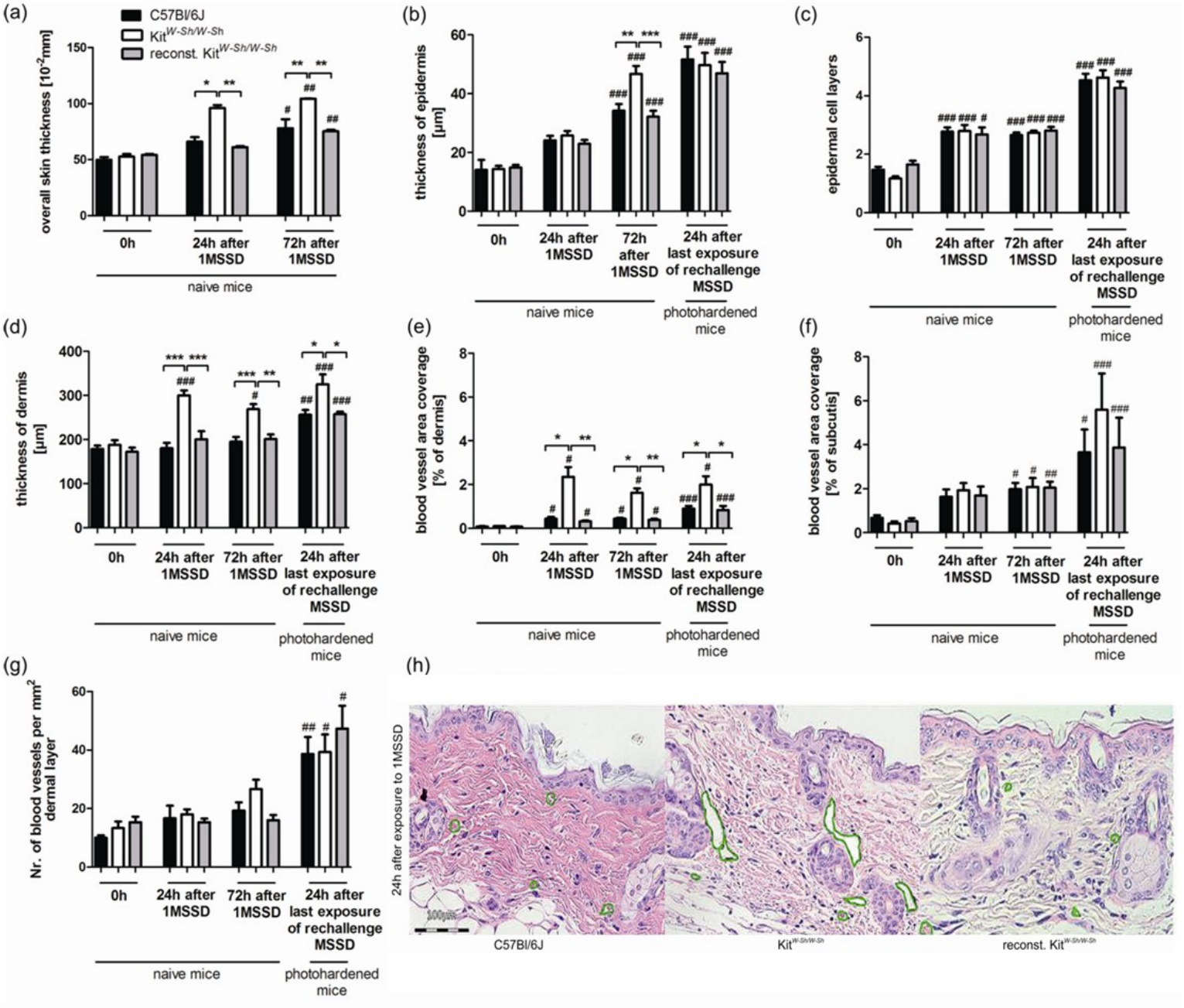

Figure 4.4.6. UV increases skin thickness and dermal blood vessel area in mast cell-deficient mice. Naive (previously unirradiated) mice were exposed to 1 MSSD as determined in Fig.4.4.4a (C57B/6J received 316 mJ/cm^2; Kit$^{W\text{-}Sh/W\text{-}Sh}$ and reconstituted Kit$^{W\text{-}Sh/W\text{-}Sh}$ received 225 mJ/cm^2) to investigate a difference in skin thickness among the mouse groups. Skin samples taken from mice 24h after last exposure of rechallenge MSSD were also investigated. (a) Double skin-fold thickness was measured 0h, 24h and 72h later with a spindle-loaded gage. (b) Thickness of epidermis, (c) number of epidermal cell layers and (d) thickness of dermis was measured in H&E-stained sections from dorsal skin of naive mice taken 0h, 24h and 72h after exposure to 1 MSSD as well as rechallenged mice. The same sections were used to quantify blood vessel area in (e) dermis and (f) sub cutis and (g) the number of blood vessels in

dermis (per mm^2). (h) Images of dermal blood vessels. Green frames indicate blood vessels which were measured in dermis and sub cutis of the three mouse groups (presented as percentage area coverage). (a-d), n = 4 mice per group. (e,f,g), n = 3 mice per group. Scale bar: 100µm. 0h = before UV irradiation. mean ± S.D. shown; * or # $P < 0.05$; ** or ## $P < 0.01$; *** or ### $P \leq 0.001$; * depicts Kruskal-Wallis test with Dunn's correction where groups were compared as indicated; # depicts Friedman test with Dunn's correction comparing individual groups vs. 0h.

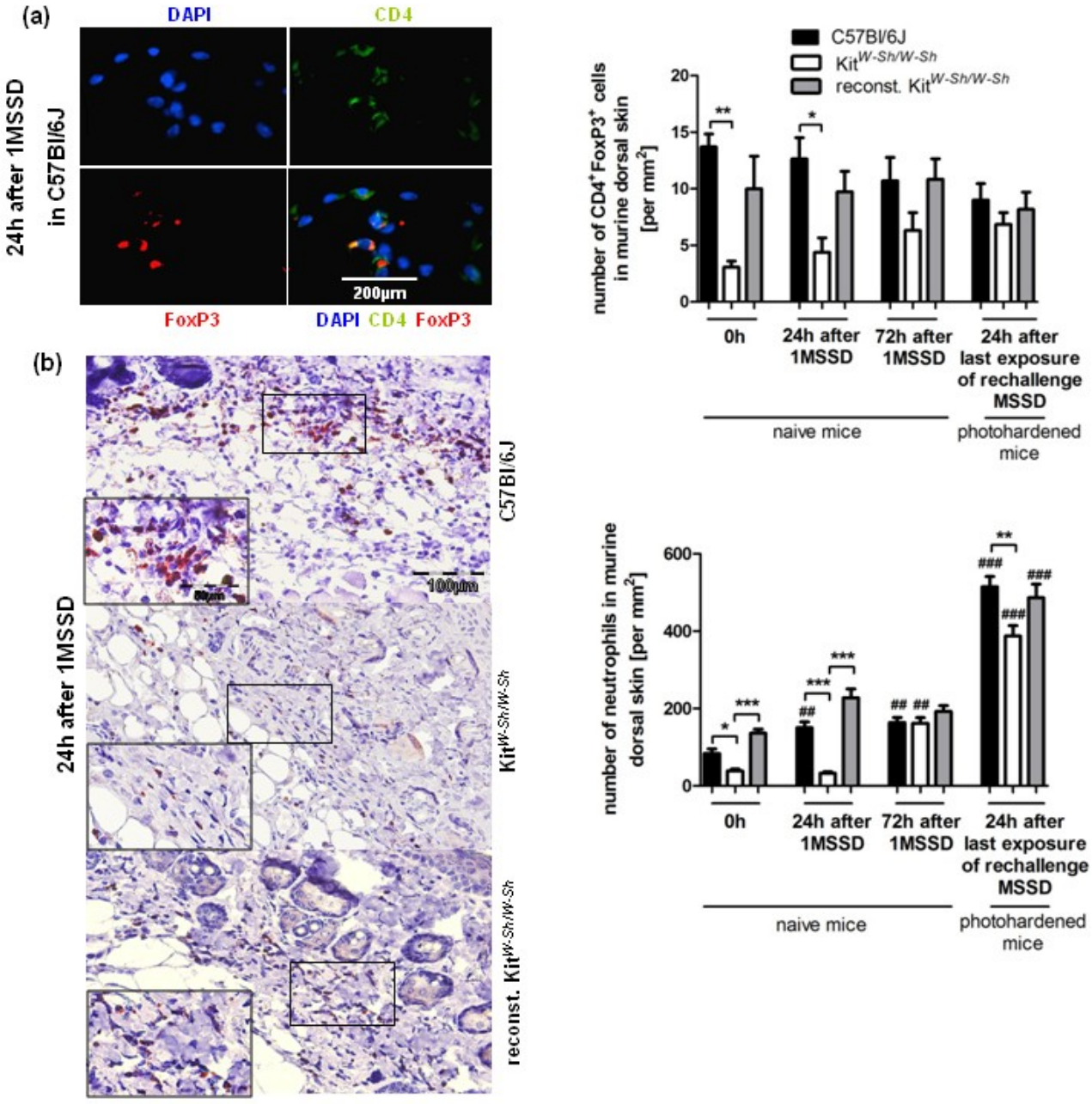

Figure 4.4.7. UV exposure of Kit$^{W\text{-}Sh/W\text{-}Sh}$ mice fails to induce cutaneous Tregs or recruit neutrophils into the skin (a) Immunofluorescent staining of Tregs in dorsal skin of naive (not photohardened) mice 0h, 24h and 72h after irradiation with 1 MSSD as well as skin from photohardened mice 24 after last exposure to rechallenge MSSD. CD25 (green), FoxP3 (red) and 4',6-Diamidino-2-phenylindole (DAPI) (blue) was used as a counterstain. Treg density was calculated per 40x powerfield. (b) Neutrophil infiltrate in dorsal skin at the same time points. (a,b), n = 3 mice per group. 0h = before UVB irradiation. Mean ± S.E.M. shown; * $P <$ 0.05; ** or ## $P < 0.01$; *** or ### $P < 0.001$; * depicts Kruskal-Wallis test with Dunn's correction where groups were compared as indicated; # depicts Friedman test with Dunn's correction comparing individual groups vs. 0h.

4.5 TABLES

Table 4.5.1. Summary of irradiation regimes

Day	UV regime
1 - 8	Pre hardening UV regime: daily UV exposure for MSSD/MPID determination
9 - 13	UV break (no exposure)
14 - 38	Photohardening UV regime (UV exposure given twice a week for 4 weeks)
39 - 41	UV break (no exposure)
42 - 50	Post hardening UV regime: daily UV exposure for rechallenge MSSD/MPID determination

Mice were irradiated with different UV regimes to determine their MSSD and relative MPID in order to investigate a potential change in sensitivity to UV after a photohardening treatment.

Table 4.5.2. Summary of MSSD and relative MPID of the different mouse groups

Experimental Group	MSSD [mJ/cm^2]			relative MPID [mJ/cm^2]		
	Pre hard-ening	Post hardening	Factor	Pre hard-ening	Post hardening	Factor
C57Bl/6J	316	442	~ 1,4	> 316	> 316	n.a.
Kit$^{W\text{-}Sh/W\text{-}Sh}$	225	225	1	59	42	~ 0,7
reconst. Kit$^{W\text{-}Sh/W\text{-}Sh}$	225	442	~ 2	> 316	> 316	n.a.

Mice were subjected to different irradiation regimes (Fig.4.4.3) and their MSSD (Fig.4.4.4a and b) and relative MPID (Fig.4.4.4c and d) are summarized in this table. MSSD, minimal skin-swelling dose; MPID, minimal photo-itch dose; n.a., not available.

5. THESIS CONTRIBUTIONS

Nina Schweintzger wrote the dissertation and drafted the figures presented in here.

Contribution to the thesis parts

- **Thesis Part 1: The effect of natural and medical photohardening on Treg levels and function in PLE patients**

Parts of this section were published in the British Journal of Dermatology (Br J Dermatol. 2015 May 30. doi: 10.1111/bjd.13930.)

Levels and function of regulatory T cells in patients with polymorphous light eruption: relation to photohardening

N. Schweintzger, A. Gruber-Wackernagel, E. Reginato, I. Bambach, F. Quehenberger, S. N. Byrne and P. Wolf

N.A.S. performed the major part of the experimental work for the Figures and Tables shown. E.R. helped with harvesting cells and added thymidine to five proliferation assays (Fig.3.4.4). I.B. contributed to the experimental work by isolating RNA from a couple of patients for RT-PCR (Fig.3.4.3). A.G.-W. recruited the patients, enrolled them in the study and provided the blood samples. F.Q. provided the statistical sample size calculation of the study and statistical analysis of Fig.3.4.3b and advised N.S. in performing the statistical analysis of the other data. S.N.B. and P.W. designed and supervised the research study. N.S. drafted the paper. All authors approved of the submitted versions and final version of the manuscript.

- **Thesis Part 2: Influence of season on vitamin D levels, Treg numbers and functions in PLE patients**

Nina Schweintzger performed the major part of the experimental work for the Figures and Tables shown. Nitesh Shirsath performed eight times the Treg staining protocol for flow cytometry (Fig. 4.4.2) and helped five times in plating of cells for proliferation assays

(Fig.4.4.7b and Fig.4.4.8b). Eleonora Reginato and Pablo Vieyra Garcia helped two times with plating of cells for proliferation assays. Alexandra Gruber-Wackernagel recruited the patients, enrolled them in the study and provided the blood samples. The determination of vitamin D 25(OH)D serum levels were done in the laboratory of Dr. Barbara Obermayer-Pietsch, Division of Endocrinology and Metabolism, Department of Internal Medicine, and blood counts were done at Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz. Franz Quehenberger performed the posthoc power analysis of the study and advised Nina Schweintzger in performing the statistical analysis of the other data. Peter Wolf designed and supervised the research study.

- **Thesis Part 3: Role of mast cells in phototolerance induction and photo itch**

Parts of this section were published in Experimental Dermatology (Experimental Dermatology 2015, DOI: 10.1111/exd.12687)

Mast cells are required for phototolerance induction and scratching abatement

Nina A. Schweintzger, Isabella Bambach, Eleonora Reginato, Gerlinde Mayer, Alberto Y. Limón-Flores, Stephen E. Ullrich, Scott N. Byrne, and Peter Wolf

N.A.S. performed the major part of the experimental work for Table 5.5.1 and 5.5.2 and the Figures displayed in this work. I.B. helped during posthardening UV regime (Fig.5.4.4b) with recording of scratching bouts of mice and assisted with mice labeling in the first week of prehardening regime (Fig.5.4.4a). E.R. assisted in sacrifice of mice after posthardening UV regime (Fig.5.4.4b). G.M. provided the Giemsa solution and the protocol for the mast cell staining (Fig.5.4.2) and the buffers for the Treg- and neutrophil staining (Fig.5.4.7a and b) together with the vegetable steamer used for the stainings. N.A.S. analyzed the data. S.N.B. and A.Y.L.-F. performed the experiment that provided the video of the paper (which cannot be shown in this work). S.E.U., S.N.B., and P.W. designed and supervised the research study and exchanged thoughts for the project. N.A.S. drafted the paper. All authors approved of the submitted versions and final version of the manuscript.

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I would also like to thank my group members Isabella Bambach, Gerlinde Mayer, Eleonora Reginato, Nitesh Shirsath, Pablo Vieyra-Garcia and Vijaykumar Patra for their incessant support and help throughout my thesis work as well as for sharing funny moments in the lab.

The most important thing during my thesis work was to know that my family will support me unconditionally, whatever may happen. Thanking you doesn't do the job at all, but it's a beginning.

Special thanks to Markus, who will make me forget a day of disappointments, anger and frustration within a second. I can't tell you how much I appreciate having you at my side.

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Declaration

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Date *Signature*