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Regulation of ultraviolet radiation induced cutaneous photoimmunosuppression by Toll-Like Receptor-4

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Abstract

UVB radiation is a potent immunosuppressive agent that inhibits cell-mediated immune responses. The mechanisms by which UVB radiation influences cell-mediated immune responses have been the subject of extensive investigation. However, the role of innate immunity on photoimmunological processes has received little attention. The purpose of this study was to determine whether toll-like receptor-4 (TLR4) contributed to UV-induced suppression of contact hypersensitivity (CHS) responses. TLR4^{-/-} and wild type C57BL/6 (TLR4^{+/+}) mice were subjected to a local UVB immunosuppression regimen consisting of 100 mJ/cm² UVB radiation followed by sensitization with the hapten DNFB. Wild type TLR4^{+/+} mice exhibited significant suppression of contact hypersensitivity response, whereas TLR4^{-/-} developed significantly less suppression. The suppression in wild type TLR4^{+/+} mice could be adoptively transferred to naïve syngeneic recipients. Moreover, there were significantly fewer Foxp3 expressing CD4⁺CD25⁺ regulatory T-cells in the draining lymph nodes of UV-irradiated TLR4^{-/-} mice than TLR4^{+/+} mice. When cytokine levels were compared in these two strains after UVB exposure, T-cells from TLR4^{+/+} mice produced higher levels of IL-10 and TGF- β and lower levels of IFN- γ and IL-17. Strategies to inhibit TLR4 may allow us to develop immunopreventive and immunotherapeutic approaches for management of UVB induced cutaneous immunosuppression.

Keywords

Toll like receptor-4; ultraviolet B radiation; contact hypersensitivity; immunosuppression

1. Introduction

Ultraviolet (UV) radiation, in particular the mid-wavelength range (UVB; 290–320 nm), is one of the most significant risk factors for the development of non-melanoma skin cancers, such as basal cell and squamous cell carcinomas. In addition to producing mutations in keratinocytes, ultraviolet radiation primarily within the UVB range (290–320 nm) is immunosuppressive. Three major lines of evidence support this statement. First, epidemiological studies indicate that 40–70% of transplant patients taking immunosuppressive medications will develop at least one non-melanoma skin cancer within ten years after having received their organ [1]. Second, exposing mice to subcarcinogenic doses of UV radiation suppresses their immune response and permits the outgrowth of

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highly antigenic UV-induced skin tumors [2, 3]. High levels of IL-10 and regulatory T-cells are important mediators of UV-induced immune suppression [3–9], and this is fully reversible by IL-12 [10, 11]. Finally, in humans, UV-induced suppression is genetically determined and those individuals in whom UV radiation does suppress cell-mediated immunity have an increased risk of developing skin cancers [12]. Analysis of the mechanisms by which UVB radiation causes skin cancer have relied to a great extent on animals, and, in particular have employed murine contact and delayed type hypersensitivity as prototypic cutaneous T-cell-mediated immune responses [5, 13–15]. Using these model systems, it has been shown that prior treatment with UVB inhibits development of T-cell mediated immune responses to topical and intradermal antigens. An essential component the UVB immunosuppressive is the generation of regulatory T-cells (2,4,5,7–9), which are, at least in part, CD4⁺, CD25⁺ T-cells and secrete large amounts of IL-10 [16].

Toll-like receptors (TLRs) have emerged as a key component of the innate immune system. They contribute to host defenses by recognizing invading pathogens and activating potentially beneficial antimicrobial inflammatory and adaptive immune responses [17]. One of these receptors, TLR4, is important because it recognizes lipopolysaccharide present in the cell wall of gram negative bacteria. Recent studies have shown that a variety of nonbacterial agonists such as taxol [18], fibronectin [19, 20], and heat shock protein 60 [21] also initiate TLR4 signaling, suggesting that it is important for non-infectious diseases as well. Toll-like receptors (TLRs) also play a role in the cellular response to UV exposure [22]. Secreted stress signals are transmitted via Toll-like receptors, thus activating the Toll–IL-1 receptor signaling cascade and the subsequent elaboration of IL-10 and TNF- α [22].

With respect to UV radiation, *Lps^d* allele has been shown be required for UV-induced immunosuppression [23]. Specifically, mice with the *Lps^d* allele were more sensitive to UVB irradiation than the mice, which lacked this allele [24]. This allele was later found to be homologous to TLR4. The purpose of this study was to delineate the role of TLR4 in UVB-induced immunosuppression using TLR4^{−/−} mice. The results could provide valuable insight into the molecular basis for TLR4-mediated photoimmunosuppression.

2. Material and Methods

2.1. Animals and Reagents

Wild-type female C57BL/6 (TLR4^{+/+}) mice 6–8 weeks of age were purchased from National Cancer Institute (Frederick, MD). TLR4^{−/−} mice on a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). All animal procedures were performed according to National Institutes of Health guidelines under protocols approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. The 1-fluoro-2, 4-dinitrobenzene (DNFB) was purchased from Sigma Chemical Co. (St. Louis, MO). Magnetic beads for purification of CD4⁺CD25⁺ T-cells were purchased from Miltenyi Biotec (Auburn, CA). Anti-mouse CD25 APC antibody was purchased from eBioscience (San Diego, CA), and anti-mouse Foxp3 PE antibody for flow cytometry was purchased from BD Pharmingen (San Diego, CA).

2.2. UVB Light Source and Irradiation of Mice

The clipper-shaved dorsal skin of mice was exposed to UVB radiation (100 mJ/cm²) from a bank of four UVB lamps (Daavlin, UVA/UVB Research Irradiation Unit, Bryan, OH) equipped with an electronic controller to regulate UVB dosage at the fixed distance of 24 cm from the lamps to the dorsal skin surface of the mice. Wavelengths <290 nm were filtered out using Kodacel cellulose film (Eastman Kodak Co., Rochester, NY). The majority of the

resulting wavelengths were in the UVB (290–320 nm; ~80%) and UVA (~20%) range, with peak emission at 314 nm as monitored regularly.

2.3. UV Induced Local Immunosuppression

UV radiation is known to cause immunosuppression which can be seen when the hapten DNFB is applied to the irradiated skin [25]. The shaved dorsal skin of TLR4^{-/-} and TLR4^{+/+} mice were exposed to UVB radiation (100mJ/cm²) for four successive days on the shaved back to induce immunosuppression in mice as described earlier with some modifications [26]. During UVB irradiation, the ears of mice were protected from UVB using opaque black tape, which was removed after exposure. One day later, the mice were treated topically with 50 µl of 0.5% DNFB in acetone/olive oil (4:1, v/v) at the UVB-irradiated site. The contact hypersensitivity response was elicited 5 days later by challenging both surfaces of the ears of each mouse with 20 µl of 0.2% DNFB in acetone/olive oil (4:1, v/v). The ear thickness was measured 24 hours after the challenge using an engineer's micrometer (Mitutoyo, Tokyo, Japan) and was compared with the ear thickness just before the challenge. Mice that received the same treatment with DNFB but were not UVB irradiated served as positive controls. Non-UVB-irradiated mice that received only ear challenge without sensitization with DNFB served as negative controls.

2.4. Adoptive Transfer of Immunosuppression

Donor mice were UVB irradiated as described earlier [16]. After 5 days, mice were sacrificed and spleens and lymph nodes were removed and CD4⁺CD25⁺ T-cells were isolated from them. 200 µl medium containing 1×10^6 CD4⁺CD25⁺ cells was injected intravenously into the recipient mice, which were then sensitized 24h later. After 5 days mice were challenged on the ear as described above.

2.5. Purification of T-Cell Populations and Stimulation for Cytokine Production

CD4⁺CD25⁺ regulatory T-cells and effector T-cells were purified from draining lymph nodes and spleens of mice by magnetobead separation using MACS column following manufacturer's instructions (Miltenyi Biotech, Auburn, CA). Purification of T-cell subpopulations was determined by flow cytometric analysis using specific antibodies to target cells. The efficiency of purification was >95%. The cells (2×10^5 in 200µl) were cultured in anti-CD3 antibody coated 96 well plates. The plates were incubated with anti-CD3 antibody (25µg/ml; 30µl/well) at 37°C for 90 min and then washed with PBS before adding the cell suspensions. Supernatants were harvested 48h later for quantification of cytokines by enzyme linked immunosorbent assay (ELISA) kits from Invitrogen (Carlsbad, CA) using standard protocols.

2.6. RNA Extraction and Real-time RT-PCR

Total RNA from CD4⁺CD25⁺ regulatory T-cells was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The concentration of total RNA was determined by measuring the absorbance at 260 nm using a BioRad Smart Spec spectrophotometer. Purity of isolated RNA was determined with the ratio of absorbance 260nm/280nm >1.9. cDNA was synthesized from 1 µg RNA using Reverse transcriptase kit (Promega) according to the manufacturer's instructions.

Using iQTM SYBR Green Master Mix (Bio-Rad, Hercules, CA), cDNA was amplified by real-time PCR with a Bio-Rad MyiQ thermocycler and SYBR Green detection system (Bio-Rad, Hercules, CA) [27]. The primers used for the following genes have been described: Foxp3 [28]. CD25 Forward 5'-ACTCCCATGACAAATCGAGAAAG-3' and reverse 5'-TCTCTTGGTGCATAGACTGTGT-3' (accession no. NM_008367); IL-10 Forward 5'-

GCTCTTACTGACTGGCATGAG-3' and Reverse 5'-CGCAGCTCTAGGAGCATGTG-3' (accession no. NM_010548) were retrieved from Primer Bank [29]. The standard PCR conditions were 95°C for 10 min and then 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The expression levels of genes were normalized to the expression level of the GAPDH mRNA in each sample. For mRNA analysis, the calculations for determining the relative level of gene expression were made using the cycle threshold (C_t) method. The mean C_t values from duplicate measurements were used to calculate the expression of the target gene with normalization to a housekeeping gene used as internal control and using the formulae $2^{-\Delta\Delta C_t}$.

2.7. Statistical Analysis

The differences between experimental groups for CHS and cytokine ELISA were analyzed using the two-tailed Student's t-test. In all cases, a $p < 0.05$ was considered significant.

3. Results

3.1. TLR4 Causes UVB-Induced Local Immunosuppression

Initial studies were conducted to determine whether TLR4 was responsible for UVB-induced immunosuppression using TLR4^{-/-} mice. We found that in WT C57BL/6 (TLR4^{+/+}) mice, as expected, the development of DNFB contact hypersensitivity was suppressed (74%) by UVB exposure (Figure 1A). The DNFB contact hypersensitivity response in TLR4^{-/-} mice was comparable to TLR4^{+/+} mice. However, there was no significant difference in the contact hypersensitivity response to DNFB in UVB treated TLR4^{-/-} mice (22%) compared to non-UVB treated TLR4^{-/-} mice (Figure 1A). Histological analysis of the ear samples further confirmed these findings (Figure 1B). These data indicate that TLR4 was responsible for UVB induced immunosuppression in mice.

3.2. Cytokine production by T-cell response in UVB induced immunosuppression

The cytokine profile of T-cells was examined after UVB induced immunosuppression. T-cells were purified from draining lymph nodes of TLR4^{+/+} and TLR4^{-/-} mice that had been UVB-irradiated and treated with DNFB. The cytokines were then examined by ELISA (Figure. 2). There were significantly high levels of IFN- γ and IL-17 in TLR4^{+/+} mice which decreased significantly after exposure to UVB radiation. In contrast, there was no significant decrease in IFN- γ and IL-17 in TLR4^{-/-} mice (Figure 2 A&B). Furthermore, there was an increase in IL-10 and TGF- β levels after exposure to UVB radiation in TLR4^{+/+} mice, but these levels remain unchanged in TLR4^{-/-} mice (Figure 2 C&D). The levels of IL-10 and TGF- β levels after exposure to UVB radiation in TLR4^{-/-} mice remain unchanged. This decrease in IFN- γ and IL-17 levels in TLR4^{+/+} mice may be attributed to the elevated levels of IL-10 and TGF- β after UVB exposure.

3.3. Phenotypic characterization of CD4⁺CD25⁺ regulatory T-cells and cytokine production

There is accumulating evidence that UV-induced regulatory T cells belong to the CD4⁺CD25⁺ T cell subset [2, 16, 30]. We wanted to investigate the levels of Foxp3 which is an important transcription factor of CD4⁺CD25⁺ regulatory T-cells. We found higher expression of Foxp3 in CD4⁺CD25⁺ regulatory T-cells from TLR4^{+/+} mice and lower expression in CD4⁺CD25⁺ regulatory T-cells from TLR4^{-/-} mice. Similarly, CD25 expression was higher in CD4⁺CD25⁺ regulatory T-cells from TLR4^{+/+} mice and lower in CD4⁺CD25⁺ regulatory T-cells from TLR4^{-/-} mice (Figure 3A). Further more, flow cytometric analysis indicated a significant increase in the number of CD25⁺ and Foxp3⁺ cells in CD4⁺ T-cells from TLR4^{+/+} mice. There was no significant difference in number of CD25⁺ and Foxp3⁺ cells in CD4⁺ T-cells from TLR4^{-/-} mice (Figure 3B). The CD4⁺ T-

cells obtained were >95% pure and there was no difference in terms of expression and percent purity between the two groups. These results indicate that TLR4 causes an increase in number of CD4⁺CD25⁺Foxp3⁺ regulatory T-cells after exposure to UVB radiation.

The cytokine profile of CD4⁺CD25⁺ regulatory T-cells revealed an increase in IL-10 and TGF- β levels after UVB exposure in TLR4^{+/+} mice. There was no significant increase in the levels of IL-10 and TGF- β in TLR4^{-/-} mice after exposure to UVB radiation (Figure 4).

3.4. UVB-irradiated immunosuppression can be adoptive transferred by CD4⁺CD25⁺ T cells in TLR4^{+/+} mice

Photoimmunosuppression is mediated primarily *via* regulatory T cells (Treg) by several other investigators [2, 4, 5, 8, 13, 31] and can be adoptively transferred in mice by CD4⁺CD25⁺ regulatory T-cells [32, 33]. The CHS response was used to determine whether CD4⁺CD25⁺ regulatory T-cells from UVB-irradiated mice could alter DNFB specific immune responses. As described in materials and methods section, 1.0×10^6 CD4⁺CD25⁺ regulatory T-cells were adoptively transferred via tail-vein injection into naive TLR4^{+/+} mice. Mice were sensitized to the hapten (DNFB) on the shaved back 24 hours after cell transfer. After 5 days, the ear-swelling response was measured by painting the ears with DNFB. Irradiation of mice with UVB significantly suppressed the ear-swelling response by 60% as compared with responses observed in non-irradiated control mice (Figure. 5A). Transfer of regulatory T-cells (1.0×10^6) decreased the responses to 52% in TLR4^{+/+} mice compared to only 25% in TLR4^{-/-} mice. These findings were in agreement with the histological analysis of ears showing varying degrees of ear thickness between the groups. These results indicate that TLR4 induced photoimmunosuppression is mediated by CD4⁺CD25⁺ regulatory T-cells and can be adoptively transferred in mice.

4. Discussion

Ultraviolet radiation primarily within the UVB range (290–320 nm) is immunosuppressive. With respect to UV radiation, TLR4 has been shown to be required for UV-induced immunosuppression. Specifically, we have found that UVB irradiation is not an effective immunosuppressive agent in TLR4^{-/-} mice that have a mutation in the TLR4 gene, although C57BL/6 in which the TLR4 gene is normal do have a suppressed response. This is consistent with the previous finding that UVB resistance correlates with the presence of *Lps^d* allele [24]. They found that C3H/HeN mice were UVB-sensitive and the C3H/HeJ mice (which lack *Lps^d* allele) were UVB-resistant strongly suggesting that this allele is important in the pathogenesis of harmful effects of UVB radiation on DNFB induced contact hypersensitivity responses. A point mutation in *Tlr4* gene, which encodes TLR4 was thought to be responsible for *Lps* hyporesponsiveness in C3H/HeJ mice (*Lps^d*) [34–36]

An essential component the UVB immunosuppression is the generation of regulatory T-cells [2, 4, 5, 7–9], which are, at least in part, CD4⁺CD25⁺ T-cells and secrete large amounts of IL-10 [16]. Interestingly, it has been reported that selected strains of mice are resistant to UV-induced immune suppression, despite the fact that they do develop antigen specific regulatory T-cells [33]. Our studies indicate that there were fewer CD4⁺CD25⁺ T-cells in TLR4^{-/-} mice than TLR4^{+/+} mice. This may be a possible reason for no upregulation of immunosuppressive cytokines IL-10 and TGF- β in TLR4^{-/-} mice. Also, the unresponsiveness to hapten DNFB could be adoptively transferred in TLR4^{+/+} mice but not in TLR4^{-/-} mice by CD4⁺CD25⁺ T-cells. Hapten-specific unresponsiveness has been reported to be adoptively transferred, because injection of lymph node cells and splenocytes obtained from UV-tolerized mice into syngeneic naive mice inhibited sensitization against the respective hapten in the recipients [37].

There is convincing evidence that IL-10 enhances the development and growth of UV-induced tumors. Because CD4⁺ UV-induced regulatory T cells produce IL-10 upon stimulation, it was suggested that IL-10 has a regulatory function. UV radiation is able to inhibit the antigen presenting function directly via UV-induced cytotoxicity as well as indirectly via the release of immunosuppressive cytokines such as IL-10 [30, 38]. From several reports it was concluded that UV-induced expression of IL-10 contributes to the development of photocarcinogenesis by suppressing protective cellular immune responses.

It is well known that IL-10 can influence Th1 cell activation by altering antigen presentation and suppressing IFN- γ secretion [39]. This was also evident in our study where we found suppression of IFN- γ in TLR4^{+/+} mice but not in TLR4^{-/-} mice. The role that UVB radiation plays in the induction of IL-17 producing T-cells has received little attention, but in this study we observed that IL-17 production by T-cells was suppressed in TLR4^{+/+} mice but not in TLR4^{-/-} mice. Additional experiments will need to be conducted to specifically evaluate the role of IL-17 in UVB-induced immunosuppression.

Photoimmunological perturbations have been exploited to treat a variety of immunologically mediated cutaneous diseases [40, 41], but have also been shown to facilitate the growth and development of UV-induced non-melanoma skin cancers [42]. Immunohistochemical studies have shown that CD4⁺CD25⁺Foxp3⁺ T-cells can be found intermingled with human basal cell carcinoma along with the Th2 cytokines IL-4 and IL-10. In the tumor microenvironment of cutaneous squamous cell carcinomas, increased IL-10, TGF- β and VEGF-A can be observed (38).

There is some controversy in the literature as to whether ligation of TLR4 *in vitro* affects the suppressive function of CD4⁺CD25⁺ T cells. However, in a recent study, chronic triggering of TLR4 through induced transgenic expression of the heat shock protein gp96 increased the regulatory activity of purified CD4⁺CD25⁺ cells. These findings illustrate that gp96 plays this role by means of its ability to chaperone TLR4 [43]. Further studies are going on in our laboratory to evaluate the mechanism through which TLR4 causes generation of CD4⁺CD25⁺ T cells in response to UVB radiation.

The pathogenic consequences of immunosuppression caused by UVB radiation can be observed in the exacerbation of infectious diseases and the development of skin cancer. Photo immunosuppression is of great clinical importance in immunocompromised patients. Transplant recipients have a highly increased risk for the development of squamous cell carcinomas on areas of sun-exposed skin. In many of the skin lesions DNA of human papilloma virus (HPV) is present, suggesting that UVB light suppresses the local immune response and renders the skin unable to reject such infectious organisms (44). Our findings indicate that TLR4 is involved in causing immunosuppression caused by UVB radiation. This will help us to identify individuals at increased risk for skin cancer and we may be able to develop inhibitors that can suppress the expression and function of TLR4. These inhibitors can be used in combination with existing agents like sunscreens.

5. Conclusion

Our observations support the concept that TLR4 mediates UVB induced suppression of cell mediated responses, primarily by the generation of CD4⁺CD25⁺Foxp3⁺ regulatory T-cells. These cells secrete large amounts of IL-10 and TGF- β and cause suppression of immune responses. Efforts to diminish TLR4 will decrease the generation of regulatory T-cells and may prove fruitful for the prevention and/or therapy of cutaneous photoimmunosuppression.

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7. References

- [1]. Kovach BB, Sams HH, Stasko T. Systemic strategies for chemoprevention of skin cancers in transplant recipients. *Clin. Transplant.* 2005; 19:726–34. [PubMed: 16313317]
- [2]. Fisher MS, Kripke ML. Suppressor T lymphocytes control the development of primary skin cancers in ultraviolet-irradiated mice. *Science.* 1982; 216:1133–1134. [PubMed: 6210958]
- [3]. Kripke ML. Antigenicity of murine skin tumors induced by ultraviolet light. *J. Natl. Cancer Inst.* 1974; 53:1333–1336. [PubMed: 4139281]
- [4]. Schwarz A, Maeda A, Wild MK, Kernebeck K, Gross N, Aragane Y, Beissert S, Vestweber D, Schwarz T. Ultraviolet radiation-induced regulatory T cells not only inhibit the induction but can suppress the effector phase of contact hypersensitivity. *J. Immunol.* 2004; 172:1036–1043. [PubMed: 14707077]
- [5]. Elmetts CA, Bergstresser PR, Tigelaar RE, Wood PJ, Streilein JW. Analysis of mechanism of unresponsiveness produced by haptens painted on skin exposed to low dose ultraviolet radiation. *J. Exp. Med.* 1983; 158:781–794. [PubMed: 6193234]
- [6]. Rivas JM, Ullrich SE. Systemic suppression of DTH by supernatants from UV irradiated keratinocytes: an essential role for interleukin-10. *J. Immunol.* 1992; 149:3865–3871. [PubMed: 1460278]
- [7]. Fisher MS, Kripke M. Further studies on the tumor-specific suppressor cells induced by ultraviolet radiation. *J. Immunol.* 1978; 121:1139–1144. [PubMed: 308520]
- [8]. Schwarz T. 25 years of UV-induced Immunosuppression Mediated by T Cells; From Disregarded T Suppressor Cells to Highly Respected Regulatory T Cells. *Photochem. Photobiol.* 2008; 84:10–18. [PubMed: 18173696]
- [9]. Moodycliffe AM, Nghiem D, Clydesdale G, Ullrich SE. Immune suppression and skin cancer development: regulation by NKT cells. *Nat. Immunol.* 2000; 1:521–525. [PubMed: 11101875]
- [10]. Schwarz A, Grabbe S, Aragane Y, Sandkuhl K, Riemann H, Luger TA, Kubin M, Trinchieri G, Schwarz T. Interleukin-12 prevents ultraviolet B-induced local immunosuppression and overcomes UVB-induced tolerance. *J. Invest. Dermatol.* 1996; 106:1187–1191. [PubMed: 8752655]
- [11]. Schmitt DA, Owen-Schaub L, Ullrich SE. Effect of IL-12 on immune suppression and suppressor cell induction by ultraviolet radiation. *J. Immunol.* 1995; 154:5114–5120. [PubMed: 7730617]
- [12]. Yoshikawa T, Rae V, Bruins-Slot W, van der Berg J-W, Taylor JR, Streilein JW. Susceptibility to effects of UVB radiation on induction of contact hypersensitivity as a risk factor for skin cancer in man. *J. Invest. Dermatol.* 1990; 95:530–536. [PubMed: 2230216]
- [13]. Toews GB, Bergstresser PR, Streilein JW, Sullivan S. Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. *J. Immunol.* 1980; 124:445–453. [PubMed: 6153101]
- [14]. Cooper KD, Fox P, Neises G, Katz SI. Effects of ultraviolet radiation on human epidermal cell alloantigen presentation, initial depression of Langerhans cell-dependent function is followed by the appearance of T6-Dr+ cells that enhance epidermal alloantigen presentation. *J. Immunol.* 1985; 134:129–137. [PubMed: 3964811]
- [15]. Cooper KD, Oberhelman L, Hamilton TA, Baadsgaard O, Terhune M, LeVee G, Anderson T, Koren H. UV exposure reduces immunization rates and promotes tolerance to epicutaneous antigens in humans, relationship to dose, CD1a-DR+ epidermal macrophage induction, and Langerhans cell depletion. *Proc. Natl. Acad. Sci. USA.* 1992; 89:8497–8501. [PubMed: 1382291]
- [16]. Maeda A, Beissert S, Schwarz T, Schwarz A. Phenotypic and Functional Characterization of Ultraviolet Radiation-Induced Regulatory T Cells. *J. Immunol.* 2008; 180:3065–3071. [PubMed: 18292529]

- [17]. Takeda K, Kaisho T, Akira S. Toll like receptors. *Annu. Rev. Immunol.* 2003; 21:335–376. [PubMed: 12524386]
- [18]. Byrd-Leifer CA, Block EF, Takeda K, Akira S, Ding A. The role of MyD88 and TLR4 in the LPS-mimetic activity of Taxol. *Eur. J. Immunol.* 2001; 31:2448–2457. [PubMed: 11500829]
- [19]. Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, Chow JC, Strauss JF III. The extra domain A of fibronectin activates Toll-like receptor 4. *J. Biol. Chem.* 2001; 276:10229–10233. [PubMed: 11150311]
- [20]. Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, Miyake K, Freudenberg M, Galanos C, Simon JC. Oligosaccharides of hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med.* 2002; 195:99–111. [PubMed: 11781369]
- [21]. Ohashi K, Burkart V, Flohe S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the Toll-like receptor-4 complex. *J. Immunol.* 2000; 164:558–561. [PubMed: 10623794]
- [22]. Kang SS, Kauls LS, Gaspari AA. Toll-like receptors: applications to dermatologic disease. *J. Am. Acad. Dermatol.* 2006; 54:951–83. [PubMed: 16713451]
- [23]. Yoshikawa T, Streilein JW. Genetic basis of the effects of ultraviolet light B on cutaneous immunity. Evidence that polymorphism at the *Tnfa* and *Lps* loci governs susceptibility. *Immunogenetics.* 1990; 32:398–405. [PubMed: 2272660]
- [24]. Kurimoto I, Streilein JW. Characterization of genetic basis of ultraviolet-B light effects on contact hypersensitivity induction. *Immunology.* 1994; 81:352–358. [PubMed: 8206509]
- [25]. Penn I. Depressed immunity and skin cancer. *Immunol. Today.* 1984; 5:291–293.
- [26]. Thatcher TH, Luzina I, Fischelevich R, Tomai MA, Miller RL, Gaspari AA. Topical imiquimod treatment prevents UV-light induced loss of contact hypersensitivity and immune tolerance. *J Invest. Dermatol.* 2006; 126:821–831. [PubMed: 16439962]
- [27]. Yusuf N, Nasti TH, Meleth S, Elmets CA. Resveratrol enhances cell-mediated immune response to DMBA through TLR4 and prevents DMBA induced cutaneous carcinogenesis. *Mol. Carcinog.* 2009; 48:713–723. [PubMed: 19142898]
- [28]. Wang GY, Zhang GY, Wang Y, Hu M, Wu H, Watson D, Hori S, Alexander IE, Harris DC, Alexander SI. *J. Am. Soc. Nephrol.* 2006; 17:697–706. [PubMed: 16467443]
- [29]. Gocke AR, Cravens PD, Ben LH, Hussain RZ, Northrop SC, Racke MK, Lovett-Racke AE. T-bet regulates the fate of Th1 and Th17 lymphocytes in autoimmunity. *J. Immunol.* 2007; 178:1341–1348. [PubMed: 17237380]
- [30]. Loser K, Apelt J, Voskort M, Mohaupt M, Balkow S, Schwarz T, Grabbe S, Beissert S. IL-10 Controls Ultraviolet-Induced Carcinogenesis in Mice. *J. Immunol.* 2007; 179:365–371. [PubMed: 17579057]
- [31]. Beissert S, Hosoi J, Kühn R, Rajewsky K, Müller W, Granstein RD. Impaired immunosuppressive response to ultraviolet radiation in interleukin-10-deficient mice. *J. Invest. Dermatol.* 1996; 107:553–557. [PubMed: 8823360]
- [32]. Elmets CA, Bergstresser PR, Tigelaar RE, Wood PJ, Streilein JW. Analysis of the mechanism of unresponsiveness produced by haptens painted on skin exposed to low dose ultraviolet radiation. *J. Exp. Med.* 1983; 158:781–794. [PubMed: 6193234]
- [33]. Schwarz A, Maeda A, Wild MK, Kernebeck K, Gross N, Aragane Y, Beissert S, Vestweber D, Schwarz T. Ultraviolet radiation induced regulatory T-cells not only inhibit the induction but can suppress the effector phase of contact hypersensitivity. *J. Immunol.* 2004; 172:1038–1043.
- [34]. Polotrak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in *Tlr4* gene. *Science.* 1998; 282:2085–2088. [PubMed: 9851930]
- [35]. Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P, Malo D. Endotoxin-tolerant mice have mutations in Toll like receptor-4 (*Tlr4*). *J. Exp. Med.* 1999; 189:615–625. [PubMed: 9989976]
- [36]. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Cutting edge: Toll like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide:

- Evidence for TLR4 as the *Lps* gene product. J. Immunol. 1999; 162:3749–52. [PubMed: 10201887]
- [37]. Shimizu T, Streilein JW. Evidence that ultraviolet B radiation induces tolerance and impairs induction of contact hypersensitivity by different mechanisms. Immunology. 1994; 82:140–148. [PubMed: 8045590]
- [38]. Kaporis HG, Guttman-Yassky E, Lowes MA, Haider AS, Fuentes-Duculan J, Darabi K, Ertelt J, Khatcherian A, Cardinale I, Novitskaya I, Krueger JG, Carucci JA. Human basal cell carcinoma is associated with Foxp3+ T cells in a Th2 dominant microenvironment. J. Invest. Dermatol. 2007; 127:2391–2398. [PubMed: 17508019]
- [39]. Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW, O'Garra A. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J. Immunol. 1991; 146:3444–3451. [PubMed: 1827484]
- [40]. Parrish JA, Jaenicke KF. Action spectrum for phototherapy of psoriasis. J Invest. Dermatol. 1981; 76:359–362. [PubMed: 7229428]
- [41]. Boukamp P. Non-melanoma skin cancer: what drives tumor development and progression. Carcinogenesis. 2005; 26:1657–1667. [PubMed: 15905207]
- [42]. Fisher MS, Kripke ML. Suppressor T lymphocytes control the development of primary skin cancers in ultraviolet-irradiated mice. Science. 1982; 216:1133–1134. [PubMed: 6210958]
- [43]. Dai J, Liu B B, Ngoi SM, Sun S, Vella AT, Li Z. TLR4 hyperresponsiveness via cell surface expression of heat shock protein gp96 potentiates suppressive function of regulatory T cells. J. Immunol. 2007; 178:3219–3225. [PubMed: 17312170]
- [44]. Vermeer BJ, Hurks M. The clinical relevance of immunosuppression by UV radiation. J. Photochem. Photobiol. B. 1994; 24:149–54. [PubMed: 7965411]

Figure 1. TLR4 causes UVB induced immunosuppression in mice

(A) The development of DNFB contact hypersensitivity was significantly suppressed (**, $p < 0.01$) by UVB exposure in WT C57BL/6 (TLR4^{+/+}) mice. However, there was no difference in the contact hypersensitivity response to DNFB in UVB treated TLR4^{-/-} mice compared to non-UVB treated TLR4^{-/-} mice. There were 5 mice per group and experiment was repeated twice and results are expressed as mean $\pm$ SEM from both experiments. (B) Histology of ear sections from day 2 after DNFB application on ear as described in the immunosuppression protocol in materials and methods section. Paraffin sections were stained with H & E and pictures were taken to show the ear swelling in corresponding groups. Bar = 100uM. The experiments were repeated twice with similar results.

Figure 2.

Cytokine profiles of T-cells purified from the draining lymph nodes from TLR4^{+/+} and TLR4^{-/-} mice. Upon UVB treatment more IL-10 (*, $p < 0.05$); and TGF- β (**, $p < 0.01$) was produced by T-cells and there was decrease in IFN- γ and IL-17 levels (**, $p < 0.01$) in TLR4^{+/+} mice. There was no significant change in the cytokine profile from T-cells from TLR4^{-/-} mice. Samples were pooled from each group with three mice and were run in duplicates and results are expressed as mean $\pm$ SD.

Figure 3.

(A) Foxp3 and CD25 expression in CD4⁺CD25⁺ regulatory T-cells. CD4⁺CD25⁺ regulatory T-cells were purified from the draining lymph nodes from TLR4^{+/+} and TLR4^{-/-} mice by magnetobead separation as described in the method section. RNA was isolated from CD4⁺CD25⁺ regulatory T-cells and Foxp3 and CD25 gene expression was determined by qPCR using custom primers as described in the methods section. (i) Expression of transcription factor Foxp3 was significantly higher (*, $p < 0.05$) in CD4⁺CD25⁺ regulatory T-cells from TLR4^{+/+} mice than TLR4^{-/-} mice. (ii) Higher levels of CD25 significantly higher (*, $p < 0.05$) in CD4⁺CD25⁺ regulatory T-cells from TLR4^{+/+} mice than TLR4^{-/-} mice. (B) CD4⁺ T-cells were purified from the draining lymph nodes from TLR4^{+/+} and TLR4^{-/-} mice by magnetobead separation as described in the method section and stained for Foxp3 and CD25. The frequency of Foxp3⁺ and CD25⁺ cells was determined by flow cytometry. There was an increase in number of Foxp3⁺ and CD25⁺ cells in the CD4⁺ T-cell population from TLR4^{+/+} mice than TLR4^{-/-} mice. The numbers indicate the percentage of positive cells in the gated Foxp3⁺ or CD25⁺ cell population. The data are representative of three independent experiments.

Figure 4.

Cytokine profiles of CD4⁺CD25⁺ regulatory T-cells purified from the draining lymph nodes from TLR4^{+/+} and TLR4^{-/-} mice. Upon UVB treatment more IL-10 (*, $p < 0.05$) and TGF-β (**, $p < 0.01$) was produced by CD4⁺CD25⁺ regulatory T-cells in TLR4^{+/+} mice. There was no significant change in the cytokine profile from T-cells from TLR4^{-/-} mice. Samples were pooled from each group with three mice and were run in duplicates and results are expressed as mean ± SD.

Figure 5. Adoptive transfer of CD4⁺CD25⁺ T-cells leads to immunosuppression in recipient TLR4^{+/+} mice. One million CD4⁺CD25⁺ regulatory T-cells were injected in mice and 24h later sensitized as described in materials and methods. Transfer of CD4⁺CD25⁺ T-cells from UVB irradiated mice reduced the CHS response significantly (**, $p < 0.01$) in TLR4^{+/+} mice than TLR4^{-/-} mice. Moreover transfer of regulatory T-cells also suppressed the CHS responses indicating the role of regulatory T-cells in UVB induced immunosuppression. (B) Histology of ear sections from day 2 after DNFB application on ear as described in the immunosuppression protocol in materials and methods section. Paraffin sections were stained with H & E and pictures were taken to show the ear swelling in corresponding groups Bar = 100uM. The experiments were repeated twice with similar results.