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Research Article

Modulation of Immune response by Ultra-Violet Light in HLA Class-II Transgenic Mice

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Abstract

Multiple Sclerosis (MS) is a chronic and debilitating disease of the central nervous system linked to both genetic and environmental factors. Among the genetic factors, MHC, especially HLA class-II, is strongly associated with predisposition to MS. Although *in vitro* studies have helped us understand some aspects of HLA class-II association with the disease, performing in vivo analysis is necessary in order to further understand this correlation. Studying the role of class-II genes in vivo is a difficult task due to the heterogeneity of human population, the complexity of MHC, and the strong linkage disequilibrium among different class-II genes. To overcome this challenge, we generated HLA class-II transgenic mice to study the role of these molecules in MS. Among the environmental factors linked with MS, ultra violet radiation (UVR)/vitamin-D is suggested to have protective effect against the development of the disease. Indeed, genetic studies have shown that presence of susceptible HLA-Class II and decrease in UVR exposure or vitamin D levels together increase risk of MS. Therefore, this study was designed to investigate the direct effect of UVR on immune response using novel humanized HLA-class II transgenic mice. HLA-class II transgenic mice expressing MS susceptible HLA-DR2 allele were treated with different doses of UVR (0.50-3.75 kJ/day) for seven consecutive days. T-cell proliferation, immune cell sub-populations and cytokines levels were analyzed. Our results show that treatment with UVR increased levels of regulatory CD4+FoxP3+ T cells and Gr1+ CD11b+ suppressive macrophages. Thus our study indicates that UVR modulates the immune response towards a tolerogenic phenotype in HLA-transgenic mice immunized with MOG_{35-55}. Therefore, HLA class-II transgenic mice offer a novel tool to decipher the mechanism by which interaction between environmental and genetic factors play a role in predisposition and/or protection against development of MS.

Keywords: EAE/MS; Epitopes; MHC; HLA-Transgenic Mice; Neuroimmunology; Vitamin D; Ultraviolet Light; Cytokines; Regulatory cells

Abbreviations

MOG: Myelin Oligodendrocytic Glycoprotein;
EAE: Experimental Autoimmune Encephalitis;
MS: Multiple Sclerosis;
SI: Stimulation Index;
UVR: Ultra-Violet Radiation

Introduction

Multiple sclerosis (MS) is a chronic, inflammatory disease of the central nervous system (CNS). The etiology of MS is extremely complex as both genetic and environmental factors may interact in different ways to influence the outcome of disease [1-3]. In this context, the challenge is to understand the roles these factors play as well as the interconnections among them. Among the genetic factors, HLA class II haplotypes especially HLA-DR2(DR β1*1501). DQ6(DQ β1*0602), DR3(DRβ1*0301).DQ2(DQβ1*0201),and DR4(DRβ 1*0401). DQ8(DQ β1*0302)have shown he strongest association with susceptibility to MS. Previously, using HLA class II transgenic mice, we have identified disease susceptible and resistance HLA class II allele(s) using Experimental Autoimmune Encephalomyelitis (EAE), an animal model of human MS [4,5]. We showed that HLA-transgenic mice expressing MS associated HLA-DR2 molecule are susceptible to Myelin Oligodendrocyte Glycoprotein (MOG) induced EAE and develop both brain as well as spinal cord pathology, hallmarks of MS disease [6,7]. Thus, HLA class-II transgenic mice authenticate role of HLA class-II gene and MOG in MS.

A number of environmental factors, most notably sunlight/ or vitamin D and Epstein-Barr virus have been linked to MS [3]. Epidemiological studies have suggested that low sunlight exposure at high altitudes might be responsible for the increased incidence of MS observed in these regions [8]. The beneficial effect of sunlight is attributed to ultra violet photon energy, which is absorbed by chromophores in the epidermis including *trans*-urocanic acid (UCA) in the stratum corneum and DNA, tryptophan and membrane lipids of epidermal cells (predominantly keratinocytes and Langerhans cells) [9]. Absorption of UVB photons by 7 dehydrocholesterol in keratinocytes initiates the pathway of vitamin D3 synthesis, which mediates immuno-suppression through vitamin D receptor (VDR).

HLA-DR2 and UVR are two of the strongest genetic and environmental factors associated with MS, respectively. Thus, investigating the effect of UVR in transgenic mice expressing MS susceptible class II gene offers an advantage as it will allow us to analyze how the interaction between a strong genetic factor and a strong environmental factor modulates the outcome of the disease. Therefore, this study was designed to investigate the direct effect of UV light on host immune response in HLA-DR2 transgenic mice. HLA-DR2 transgenic mice were treated with different doses of UVB light and were analyzed for presence of different immune subsets, T cell proliferation and cytokine response. We observed that treatment with UVB light caused induction of immune suppressive pathways, indicating the importance of UV irradiation in the modulation of immune response.

Material and Methods

Transgenic mice

The HLA-DR2 (DRB1*1502) transgenic mice lacking endogenous MHC class II genes were produced, as previously described [10,11]. Transgene negative littermates were used as controls. All mice were bred and maintained in the pathogen free Immunogenetics Mouse Colony of Mayo Clinic according to the National Institute of Health and institutional guidelines. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC), at Mayo Clinic, Rochester.

Flow cytometry

Expression of HLA-DR molecules on peripheral blood leukocytes, lymph node cells (LNCs), and splenocytes were analyzed by flow cytometry using monoclonal antibodies (mAbs) L227 specific for HLA-DR [12]. Surface expression of CD4 (GK1.5), CD8 (53.6.72), B cells (RA3-6B2), CD11c+ DCs (HL3), CD11b+ monocytes/macrophages (M1/70), NK1.1 cells (PK136), CD25 (PC61), Ly6G/Gr-1 (RB6-8C5) were analyzed using fluorescent conjugated mAb from BD Biosciences (San Jose, USA). Frequency of FoxP3+ (FJK-16s) regulatory CD4 T cells was analyzed using Anti-Mouse/Rat FoxP3 staining kit from eBiosciences™ (San Diego, CA, USA) using manufacturer's protocol.

Peptide

Twenty-amino acid-long synthetic peptide MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) was synthesized at the peptide core facility of Mayo Clinic, Rochester, MN.

UV irradiation of the mice

To directly analyze the effect of UVR, we shaved the backs of HLA-DR2 transgenic mice and treated them with various doses of UVR using a UV lamp emitting a broad band of UVB rays ranging from 280-360 nm. The doses of UVR were standardized using an UVX radiometer equipped with a 302 nm sensor (UVP). Mice were either left untreated or treated with 0.5 (2min), 1.25 (5min), 2.5 (10min) and 5.0 (20min) KJ/m^2 of UVR once daily for 7 days to analyze effect of UVR in naïve mice. To analyze the effect of UV irradiation on antigen specific immune response, animals were irradiated as above, immunized with antigen and then treated with UVR every other day until sacrificed. These doses were chosen based on published studies [13]. All the control mice were shaved and sham treated.

Immunization and T cell proliferation assay

Mice were immunized subcutaneously with MOG_{35-55}(100 μg) peptide, emulsified in CFA (1:1) containing 100μg of Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) as described previously [6,14]. Some immunized mice were sacrificed 10 days after immunization, draining lymph nodes removed and challenged *in vitro* with antigen [6,14]. The results are presented as stimulation indices (CPM of test sample/ CPM of the control). For *in vitro* inhibition experiments, mAbs specific for CD4 (GK1.5), CD8 (TIB 105),

HLA-DQ (IVD12), and HLA-DR (L227) were added to LNCs challenged *in vitro* with human PLP_{91-110} (20µg/ml). All of the neutralizing antibodies were generated in-house using the Mayo antibody core facility.

Cytokine production

Splenocytes were collected 10 days post immunization and stimulated with MOG_{35-55} peptide as mentioned before in the T cell proliferation section. Supernatants were collected from culture 48 hrs after peptide stimulation. The concentration of cytokines (IFN-γ, IL-10, IL-17 and TNF-α) in the supernatant was measured by sandwich ELISA using pairs of relevant anti-cytokine monoclonal antibodies according to manufacturer's protocol (Pharmingen, San Deigo, California, USA).

Statistical analysis

The differences in proliferation or in cytokine levels between groups was assessed by a one-way analysis of variance with multiple comparisons of the means when more than two groups were analyzed, or by Student's t-test when only two groups were analyzed.

Result and Discussion

Effect of UV light on Immune cells

To directly analyze the effect of UVR, we shaved the backs of HLA-DR2 transgenic mice and treated them with 0.5 (2 min), 1.25 (5 min), 2.5 (10 min) or 5.0 (20 min) KJ/m^2 of UVR once daily for 7 days or left untreated as controls. After the seventh dose, mice were sacrificed; spleens were collected for cellular profiling by flow cytometry. UV irradiation had no effect on frequency of CD4 (Figure 1A) or CD8 T (Figure 1B) cell population at neither of the tested doses.

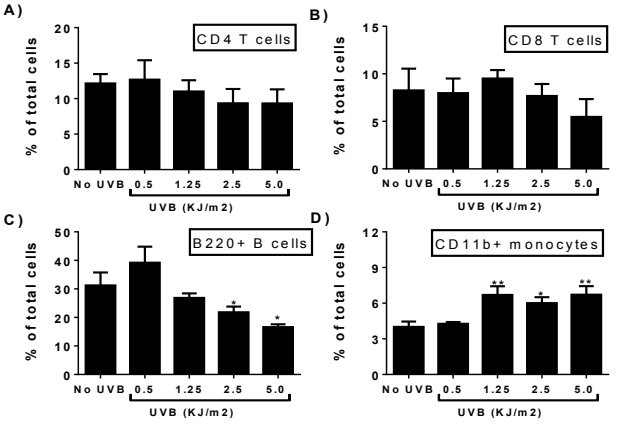

Figure 1. Effect of UVR treatment on immune cells in HLA-DR2 transgenic mice. UVR treatment had no effect on CD4 (A) or CD8 T cell (B) subsets, whereas it modulated levels of B cells (C) and CD11b+ monocytes (D) in HLA-DR2 transgenic mice. Mice were treated with different doses of UVR for one week and splenocytes were isolated for analysis of different immune subset using specific cell surface markers by flow cytometry. Numbers in histograms indicate the percentage of cells positive for the particular marker. Data represent one of three experiments performed at different time points.

However, UVR modulated frequency of B cells and monocyte populations (Figure 1C and D). While lower doses of 0.5 and 1.25 KJ/m^2 had no effect on B cell population, higher doses of 5 and 10 KJ/m^2 caused a decrease in B cell population (Figure 1C). Similarly, all doses, except the smallest dose of 0.5 KJ/m^2, caused an increase in CD11b+ population (Fig ure 1D). Our study indicates that while UVR at high doses can suppress B cell population, it caused an increase in CD11b+ monocyte population. No effect was observed on T cell populations, however.

Effect of UV light on anti-CD3/CD28 stimulated T cell proliferation

To analyze the effect of UVR on mitogen induced T cell proliferation, HLA-DR2 transgenic mice were either left untreated or treated with 0.5, 1.25, 2.5 and 5.0 KJ/m^2 of UVR once daily for 7 days as mentioned previously. After the seventh dose, mice were sacrificed; spleens were collected and cultured in anti-CD3/CD28 coated plates. Mitogen specific T cell proliferation was measured using standard tritiated thymidine (3H-TdR) incorporation assay [15]. The smallest dose of 0.5 KJ/m^2 caused an increase in T cell proliferation, whereas all other groups treated with 1.25, 5 and 5 KJ/m^2 doses showed decreased T cell proliferation compared to the untreated group (Figure 2). Treatment with the 1.25 KJ/m^2 dose caused 35±14% suppression in T cell proliferative response, whereas the maximum suppression (54±7%) was observed in the mice treated with the 2.5 KJ/m^2 dose; suppression at the highest dose of 5 KJ/m^2 was similar to the 2.5KJ/m^2 dose. Our study indicates, therefore, that UVR might be immunostimulatory at low dose, as it induced proliferation of T cells, whereas it is tolerogenic at high doses, causing suppression of T cell response.

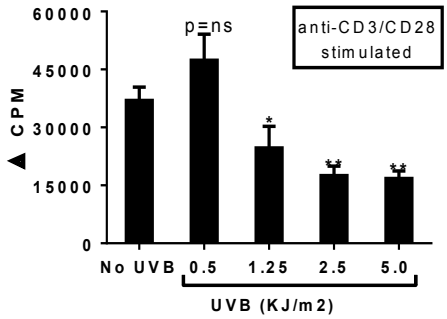

Figure 2. Effect of UVR treatment on anti-CD3/CD8 induced T cells proliferation in HLA-DR2 transgenic mice. UVR treatment suppressed, anti-CD3/CD28 stimulated T cell response in a dose dependent manner. Mice were treated with different doses of UVR for one week, and splenocytes were cultured in anti-CD3/

CD28 coated plates for 48 hrs. The proliferative response was assessed by pulsing the cultures with [^{3}H]thymidine for the last 16 h. The data are presented as the difference in CPM over medium control (Δ CPM) and are average of three independent experiments.
* p ≤0.05 and ** p≤ 0.005 as compared to untreated (no UVB) group.

UVR treatment causes suppression of pro-inflammatory cytokines and increase in anti-inflammatory cytokine IL10

Next, we measured levels of pro- and anti-inflammatory cytokines to determine, whether UVR can modulate the cytokine response. Cell free culture supernatant from anti-CD3/CD28 stimulated splenocytes culture was collected and levels of IL-10, IL-17, TNFα and IFNγ were analyzed using commercial sandwich ELISA kit. The lowest dose of UVR had no effect on levels of IFNγ (Figure 3A), however, it caused an increase in the levels of pro-inflammatory cytokines IL-17 compared to the control group (Figure 3B). Although the UVR treated groups showed a trend towards an increase in TNFα levels (Figure 3C), it did not reach a statistical significance. High UVR doses of 1.25, 2.5 and 5 KJ/m^2 caused suppression of IL-17 (Figure 3B) and TNFα (Figure 3C) but suppressed IFNγ only at doses of 2.5 and 5 KJ/m^2 (Figure 3A). The anti-inflammatory cytokine IL10 was up regulated by the two highest doses (2.5 and 5 KJ/m^2) of UVR treatment (Figure 3D). Thus, our cytokine data indicates that UVR treatment induces a tolerogenic response as it suppressed pro-inflammatory cytokines and caused an increase in levels of anti-inflammatory cytokine IL10.

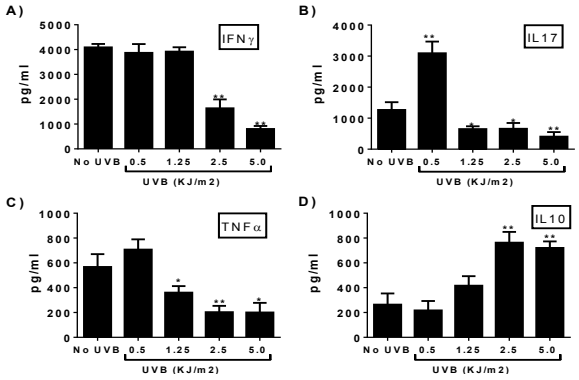

Figure 3. Effect of UVR treatment on anti-CD3/CD8 induced cytokine response in HLA-DR2 transgenic mice. UVR treatment suppressed levels of pro-inflammatory cytokines IFNγ (A), IL17 (B), and TNFα (C), whereas it caused an increased in levels of anti-inflammatory cytokine IL10 (D). Mice were treated with different doses of UVR for one week, and splenocytes were cultured in anti-CD3/CD28 coated plates for 48 hrs. Cell free supernatants were analyzed for cytokines by standard sandwich ELISA as described in material and methods. Data is presented as means ± SD of at least four different mice. * p ≤0.05 and ** p ≤0.005 as compared to untreated (no UVB) group.

UVR treatment also suppresses antigen specific T cell and cytokine response

To investigate whether UVR treatment can suppress antigen specific T cell response, mice were either left untreated or treated with various doses of UVR (as shown in Figure 4) once daily for 7 days. After the seventh dose, mice were immunized with MOG_{35-55} antigen emulsified in CFA and then treated with UVR every other day. Ten days postimmunization, mice were sacrificed; spleens were collected for T cell recall response and cytokines. Treatment with UVR suppressed antigen specific immune response in a dose dependent manner, with the exception of the lowest dose at 0.5 KJ/m^2 (Figure 4A). Maximum suppression of T cell proliferation was observed at 2.5 and 5 KJ/m^2. Next we analyzed the effect of UVR on pro and anti-inflammatory cytokines in T cell recall response. Treatment with UVR at doses of 2.5 and 5 KJ/m^2 suppressed pro-inflammatory cytokines such as IFNγ and IL17 (Figure 4B and 4C). However, UVR increased levels of IL10 indicating that it has an immunosuppressive effect (Figure. 4D). Altogether, these results show UVR can suppress antigen specific immune response in HLA class II transgenic mice.

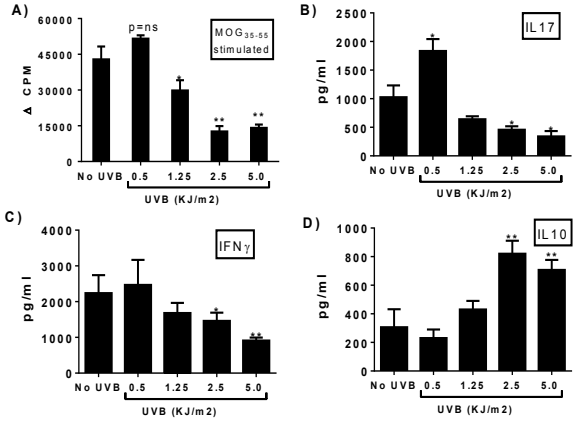

Figure 4. Effect of UVR treatment on MOG_{35-55} induced T cells proliferation in HLA-DR2 transgenic mice. A) At all doses, except 0.5 KJ/m^2, UVR treatment suppressed MOG_{35-55} stimulated T cell response in HLA-DR2 transgenic mice. Mice were treated with different doses of UVR for one week, followed by immunization with MOG_{35-55} peptide emulsified in CFA and then treated with UVR every other day. Ten days post-immunization, mice were sacrificed and spleens were collected for T cell recall response. For measurement of antigen specific T cell responses, draining LNCs from Tg mice immunized with PLP_{91-110} were cultured with or without (control) MOG peptide for 48 h. The proliferative response was assessed by pulsing the cultures with [^{3}H] thymidine for the last 16 h. The data represents the average of three independent experiments and is presented as the difference in the CPM over medium control (Δ CPM). UVR treatment suppressed levels of pro-inflammatory cytokines IFNγ (B) and IL17 (C), whereas it caused an increased in levels of anti-inflammatory cytokine IL10 (D). Cytokines level in culture supernatants were determined by standard sandwich ELISA as described in material and methods. * p ≤0.05 and ** p ≤0.005 as compared to untreated (no UVB) group.

UVR suppressed T cell proliferation by induction of suppressive macrophages and regulatory CD4 T cells

To understand the mechanism of immune-suppression by

UV light, we analyzed the cellular profile of mice treated with different doses of UVR. Splenocytes were stained with CD11b and Gr-1 or CD4, CD25 and FoxP3 to analyze levels of suppressive monocytes and regulatory T cells respectively. As shown in Figure 5A and 5B, all doses of UVR, except the 0.5 KJ/m^2 dose, caused an increase in the levels of CD11b+Gr-1+ suppressive monocytes with 5KJ/m^2 dose causing a 4 fold increase over control, whereas 2.5 KJ/m^2 dose caused a 3 fold increase compared to the untreated group. Analysis of CD4+FoxP3+T cells showed that all higher doses of 1.25, 2.5 and 5 KJ/m^2 caused an increase in the frequency of CD4+FoxP3+Treg population with 1.25 KJ/m^2 dose causing a 2 fold increase whereas 2.5 KJ/m^2 caused an increase of ~2.5 fold compared to the untreated group (Figure 5C and 5D). At the lowest dose of 0.5 KJ/m^2, UVR had no effect on Treg population. The increase in the group receiving the highest dose of 5 KJ/m^2 was similar to the 2.5 KJ/m^2 dose. Thus, our results show that at higher doses, UVR treatment can induce immune-regulatory populations.

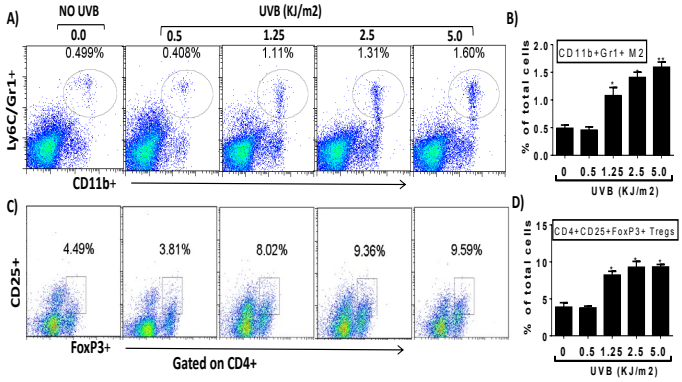

Figure 5. Effect of UVR treatment on immune-regulatory cell populations. UVR treatment led to increase in CD11b+Gr1+ suppressive monocytes as well as CD4+CD25+FoxP3+ Tregs population. Mice were treated with different doses of UVR for one week, followed by immunization with MOG_{35-55} peptide emulsified in CFA and then treated with UVR every other day. Ten days postimmunization, mice were sacrificed, and spleens were collected for flow cytometry analysis of regulatory immune subsets by staining with either α-CD25-PE, α-Foxp3-APC and α-CD4-PerCp or α-GITR-PE and α-CD11b-PerCp. The analysis was performed with FlowJo software (Tree Star, San Carlos, CA, USA). Representative dot-plot shows numbers in quadrants indicating percent cells in that gate and numbers in histograms indicate the average percentage of cells positive for the particular marker. The data are pooled from two independent experiments with minimum of four animals per group.

This study shows that UVR treatment can suppress both non-specific as well as antigen specific immune response in HLA transgenic mice expressing MS susceptible –DR2 molecule. We further show that UVR also suppresses levels of pro-inflammatory cytokines and increases levels of anti-inflammatory cytokine. Our cellular profiling data indicates that UVR might suppress T cell proliferation and pro-inflammatory cytokine through up regulation of suppressive monocytes and FoxP3+regulatory CD4 T cells. We have previously shown that HLA class-II transgenic mice are an excellent animal model to study human MS [4-6]. This is the first study to show an immunomodulatory role of UVR in the setting of HLA class II transgenic mice.

The ability of UVR to suppress immune response is in agreement with earlier reports showing that ultraviolet radiation can suppress cell mediated immune response [16,17]. UVR has been shown to suppress immune response through release of inflammatory mediators such as IL10 [18,19]. A number of mechanisms have been proposed to explain the immune-suppressive nature of UV radiation, especially regulatory CD4 T cells, NK cells and suppressive macrophages [9,19-21]. Thus, the ability of UVR to induce Tregs and suppressive macrophages is in agreement with earlier published reports. We did not find any increase in NK cells or suppressive CD8 T cells; however, this might be due to differences in mouse strains and genetic background. The fact that UVR plays a direct role in the synthesis of vitamin D has led to the theory that UVR mediates its immunosuppressive effect through vitamin D. An important role of vitamin D in MS was further supported by studies showing decreased levels of vitamin D in MS patients compared to healthy controls [6]. Vitamin-D can modulate immune response through multiple pathways including modulation of APCs, Tregs and suppressive monocyte function [22-24]. Based on these findings, vitamin D has been actively pursued as a therapeutic agent for MS treatment. Nonetheless, the role of vitamin D in MS/EAE is complicated by the observations that i) doses of vitamin D required to suppress EAE are toxic and induce hypercalcemia; and ii) mice lacking vitamin D receptor develop significantly less severe disease as compared to wild type mice [25]. Since UVR can cause immunosuppression through pathways that are both dependent and independent of vitamin D, there has been a growing interest in investigating Vitamin D- independent pathway(s) induced by UVR that result in immunomodulation of MS [9]. One of the putative molecules induced by UVR independent of vitamin D is cis-urocanic acid (cis-UCA). cis-UCA is formed in the skin by photosiomerization of trans-UCA [26] and has been shown to cause both local as well as systemic immunosuppression [27]. Although it is taken for granted that most of the effect of sunlight/UVR on MS is through induction of vitamin D and subsequent modulation of the immune response, recent reports suggest that UVR might have direct effect on the disease process independent of vitaminD [9,13]. Thus our HLA-DR2 transgenic mice can be used to decipher the role of vitamin D dependent as well as vitamin D independent pathways involved in UVR induced immunomodulation. A better understanding of these pathways will help in elucidating the mechanism by which UVR suppresses inflammatory immune response. Moreover, this will ultimately contribute to the development of novel therapeutic agents to treat MS.

Conclusion

HLA polymorphism and UVR exposure through sunlight are two important factors linked to predisposition to MS. In hu-

mans, predisposition to MS is associated with the presence of the HLA-DR2, -DR3 or -DR4 allele and UVR can modulate disease differently. Our study shows that UVR can modulate immune-response in HLA-DR2 transgenic mice. Specifically, UVR treatment – i) suppressed both antigen specific as well as mitogen specific T cell proliferation; ii) caused an increase in the frequency of suppressive monocytes and regulatory T cells; iii) inhibited production of pro-inflammatory cytokines; and iv) induced production of anti-inflammatory cytokine IL-10. Thus our study indicates that HLA class II transgenic mice can serve as an animal model of human MS to study the interaction between genetic and environmental factor in the setting of MS associated HLA-class II gene.

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