Ultraviolet B but not A Radiation Activates Suppressor B Cells in Draining Lymph Nodes

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Abbreviations:

APC, antigen presenting cell; CS, contact sensitivity; DC, dendritic cell; MHC, major histocompatibility complex; ssUV, solar simulated ultraviolet TNCB, tri-nitro-chloro-benzene; UV, ultraviolet;

ABSTRACT

Immunosuppressive doses of solar simulated ultraviolet (UV) radiation activate lymph node B cells that can suppress primary immunity by inhibiting the function of dendritic cells. The aim of this study was to determine the waveband responsible for activation of these suppressor B cells. We exposed C57BL/6 mice to various doses of either UVA or UVB radiation and analysed the number and activation state of lymph node antigen presenting cells. Immunosuppressive doses of UVB but not UVA activated B cells as assessed by MHC II expression and doubled their numbers in draining lymph nodes. Higher doses of UVA that were not immunosuppressive actually suppressed B cell activation. Our results show that UVA and UVB suppress systemic immunity via different mechanisms. Lymph node B cells are activated in response to immunosuppressive doses of UVB but not UVA. Thus the activation state of lymph node APC appears to be important for UV immunomodulation.

INTRODUCTION

Ultraviolet (UV) radiation in sunlight causes skin cancer by damaging DNA and suppressing the host immune response. While it is well recognised that the UVB (290-320nm) component of the solar spectrum can inflict both of these carcinogenic events (1), the DNA damaging (2-4) and immunosuppressive properties (5, 6) of the UVA (320-400nm) component are only now being fully appreciated. The systemic immunomodulating effects of UV are dose-dependent. We have recently shown that increasing doses of UVB up to about 1 minimal edemal dose (MED), inhibits both primary and secondary immunity in a linear dose related manner. In contrast, only low doses of UVA inhibit primary but not secondary immunity, with higher UVA doses protecting mice from UVB-induced primary immune suppression (5). This immunoprotective effect of high dose UVA had been previously reported by Reeve et al. (7). In light of these advances, it is important that we establish the precise mechanisms by which different doses of each component of the UV-solar spectrum contributes to systemic immunomodulation.

We recently demonstrated a novel mechanism of solar-simulated UV (ssUV)-induced immune suppression that involved the activation of lymph node B cells. Exposure to immunosuppressive ssUV resulted in a selective increase in the number and activation state of MHC $II^{+}B220^{+}IgD^{+}CD19^{+}CD11c^{-}$ $CD4^{-}CD8^{-}$ B cells. Confirming their important role in mediating ssUV-induced systemic immunosuppression, these B cells suppressed the activation of primary immunity when co-administered with antigen loaded lymph node dendritic cells (DC) (8). Until now, the waveband and dose of the UV-solar spectrum responsible for activating these suppressor B cells has been unknown. In this study, we show that the UVB, but not the UVA component of sunlight (although both are immunosuppressive), is responsible for activating B cells in the draining lymph nodes. In contrast,

higher doses of UVA that protected mice from immunosuppression activated lymph node DC, suggesting a possible novel mechanism of UVA-induced immunoprotection.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice (Animal Resource Centre, Perth, WA, Australia) aged 8-10 weeks at the start of irradiations were used with the approval of the Sydney University animal ethics committee.

UV Source and Irradiations

The UVA and UVB spectra used in this study were produced with a 1000W xenon arc solar simulator (Oriel, Stratford, CT) monitored with an Optronics spectroradiometer as previously described (5). The UVA and UVB spectra are shown in Fig. 1, with the irradiance. For the purposes of this study, we have defined UVA as that spectrum that contains no wavelengths below 320nm and UVB as that which does contain wavelengths between 290 and 320nm. The irradiation regime is identical to that which we have previously published (8). Briefly, mice were shaved on their dorsal trunk 24 h prior to irradiation with various doses of UVA or UVB each day for three consecutive days. During the brief irradiation (ranging from 10-60 sec) the ears and head were shielded from the UV with black Perspex. Control mice were shaved and restrained only. Three days after the final UV-irradiation, mice were either euthanased by cervical dislocation or contact sensitised.

Determination of Immunosuppression and Tolerance by Contact Sensitivity

Mice were sensitised by applying 50 µl of a 4% wt/vol solution of tri-nitro-chloro-benzene (TNCB; ICI Chemical Co. Tokyo, Japan) in 4:1 acetone:olive oil to the shaved un-irradiated abdomen three days after the final UV exposure, with positive control unirradiated mice being sensitised in the same way. To assess primary contact sensitivity (CS), 20 µl of a 1% solution of TNCB in 4:1 acetone:olive oil was applied to both sides of the right ear of the mice seven days later. After a further 24 h, the difference in thickness between the right challenged and left unchallenged ears were measured using engineers callipers (Mitutoyo Corporation, Kanagawa, Japan). The increase in ear thickness of negative control

unirradiated, unsensitised but challenged only mice (irritant control) were subtracted from the test groups.

For assessment of UV-induced secondary immunity, or tolerance, the same groups of mice were rested for 4 weeks. At this time the shaved abdomen was re-sensitised with 50 µl of 4% TNCB in 4:1 acetone:olive oil. 7 days later, 20 µl of a 1% solution of TNCB in 4:1 acetone:olive oil was applied to both sides of the left ear and the difference in thickness between the left challenged and right unchallenged ears was read 24h later.

Flow Cytometry

Each experiment (repeated 3-5 times) contained groups of 3 control and 3 UV-irradiated animals. The left and right inguinal lymph nodes from individual mice were removed and pooled. In these experiments, mice were not sensitised to antigen to examine the effect of UV irradiation only. As we have described previously (8), single lymph node cell suspensions were prepared, the cells counted using a haemocytometer and then labelled with the following antibodies (clones) for four colour flow cytometry; CD11c (HL3), I-A^b (AF6-120.1), IgD (11-26c.2a), IgM (II/41) and B220 (RA3-6B2) (all from Pharmingen; Franklin Lakes, NJ). Isotype control antibodies were used in parallel to ensure antibody specificity and produce the electronic gates for analysis of positively labelled cells. Acquisition was performed on a FACSCalibur Flow Cytometer (Becton Dickinson; BD Franklin Lakes, NJ) and populations were analysed using CellQuest Pro software (BD).

Statistical Analysis

In all experiments, an un-paired two-tailed Student's t-test was used, where $p < 0.05$ was considered statistically significant. The results from 3-5 repeat experiments were normalised against the positive

control group in each experiment and pooled for final analysis. The mathematical process of normalisation has been described by us previously (9).

RESULTS

Both UVA and UVB suppress Primary Immunity, but only UVB induces Tolerance

We have previously shown using oxazolone as the contact antigen, that UVB suppresses primary systemic CS in a linear dose related manner, while UVA suppresses systemic CS at low but not higher doses (5). We have confirmed this data here using the contact antigen TNCB (Fig. 2), although the UV doses were slightly different with the different contact sensitizer. UVA at 1200 mJ/cm^2; which corresponds to the amount of UVA found in 0.36 of the amount of solar-simulated UV that causes sunburn, but not 2400 mJ/cm^2 of UVA, causes significant immunosuppression in C57BL/6 mice (Fig. 2; left panel). The magnitude of this immunosuppression was almost identical to that caused by 100 mJ/cm^2 of UVB. If the UVA and UVB components at each dose were combined, they would form a reasonable approximation to sunlight at that dose. Higher doses of UVB caused higher levels of immunosuppression (Fig. 2). In contrast and consistent with our previous observations (5), higher doses of UVA were not immunosuppressive.

As predicted, when the mice were rested for 4 weeks and re-sensitised for assessment of UV-induced tolerance, only the UVB doses suppressed secondary immunity (Fig. 2; right panel). This result is consistent with our previous observations using oxazolone (5). Hence both UVA and UVB can suppress the induction of primary immunity at low doses, but only UVB is able to suppress the memory response.

Exposure to UVB but not UVA increases total lymph node, B cell and dendritic cell numbers

Our previous research demonstrated that exposure to an immunosuppressive dose of ssUV (UVA and UVB combined) caused a significant increase in total draining lymph node cell numbers three days after the last irradiation (8). Exposure to both doses of UVB but not UVA, in the absence of antigen, increased the total number of cells in the draining lymph nodes as well as the total number of MHC $II^+B220^+IgD^+CD11c^-$ B cells and MHC II^+CD11c^+ DC (Fig. 3). Following exposure to 100 mJ/cm^2 UVB, the B cells and DC in the DLN, when expressed as a percentage of the total lymph node cells, increased significantly above control groups by 44 ± 10% and 32 ± 10% respectively. Thus the B cells and DC increased considerably more than other cells in the lymph nodes. Hence, although both UVA and UVB caused significant primary immune suppression, only the UVB wavelengths induced changes to cell numbers, and B cell and DC percentages in the draining lymph nodes.

Immunosuppressive UVB Activates Lymph Node B cells While Immunoprotective UVA Activates Lymph Node DC

We determined whether UVB or UVA could activate lymph node B cells or DC. UVB but not UVA activated lymph node B cells, as assessed by a significant enhancement in the expression of MHC II on these cells (Fig. 4; left panel). In contrast, MHC II expression on lymph node DC was not significantly altered by exposure to UVB. A dose of UVA that caused significant primary immunosuppression (1200 mJ/cm^2) did not significantly alter the expression of MHC II on B cells or DC. Surprisingly, a higher UVA dose that did not cause primary immune suppression (2400 mJ/cm^2; Fig. 2 left panel) induced a small but significant *decrease* in expression of MHC II on lymph node B cells (Fig. 4; left panel) and a small but significant *increase* in the expression of MHC II on lymph node DC (Fig. 4; right panel). These results are consistent with our theory that the UVB portion of the solar UV spectrum is responsible for activating lymph node B cells.

DISCUSSION

Exposure to the UV wavelengths (280-400nm) in sunlight is a serious human health concern as they are the prime aetiological cause of both melanoma (10) and epithelial skin cancer (11). Skin cancer incidence and mortality rates continue to rise and the overall cost of treating skin cancer is a huge burden on government health budgets. In addition, epidemiological evidence shows that UV may also contribute to the development of certain types of leukaemia, including B cell derived non-Hodgkin's lymphoma (12, 13). Supporting these findings is the recent demonstration that exposing $p53^{+/-}$ heterozygous mice to UVB leads to the development of B cell lymphomas (14).

DNA damage in conjunction with suppression of the immune response is required for carcinogenesis. While we know that both the UVA and UVB components of sunlight cause DNA damage (15), exactly how each of these wavebands induce immune suppression is still not understood. Both the UVB and UVA wavebands of sunlight cause immune suppression, although the mechanisms involved are not clear. Understanding the precise mechanisms of how each component induces systemic immune suppression is essential for the development of successful treatment regimes. This is compounded by the complexity of UVA effects on immunity as they do not show a straight forward dose response. We have shown here that UVB but not UVA increases the number and activation state of B cells in the draining lymph nodes. The UVB source we used was not pure, but also contained some UVA and visible radiation. However the UVA source, which did not contain any UVB, but UVA with some contaminating visible radiation did not cause these changes. Therefore the lymph node changes described here were due to UVB, not UVA. UVB irradiation, in the absence of exogenously applied antigen, increased total draining lymph node cell numbers. As a result of this UVB-induced increase in total lymph node cells, the number of B cells and DC also increased. However, the percentage of B cells and DC compared to other cells in the lymph nodes also increased following exposure to UVB,

showing that there was a selective increase in these cells, and not just a general increase in all lymph node cells to the same extent. We have previously shown that these activated B cells can inhibit DC-induction of T cell immunity (8). Interestingly, although UVA suppressed primary immunity to approximately the same degree as UVB, no corresponding activation of lymph node B cells was observed. These results imply that UVA causes primary immune suppression via a different mechanism, one that probably involves the release of reactive oxygen species (16, 17). Moreover, because UVA was unable to suppress the induction of secondary immunity, it is possible that the activation of lymph node suppressor B cells is not only important for suppression of primary immunity but is also a key event in the development of immunological tolerance.

In this study, while both doses of UVB studied activated lymph node B cells, the higher dose of UVA reduced the level of MHC II on lymph node B cells. This result is particularly important considering that this higher UVA dose has previously been shown by us and others to protect UVB-irradiated mice from immunosuppression (5, 7). It is important to bear in mind that although this UVA dose has been termed "high" it is in fact equivalent to the amount of UVA contained in sunlight that would cause a barely detectable sunburn. Hence humans could easily be exposed to such UVA doses under normal every day conditions. High dose UVA-induced immune protection is mediated in part by IFNγ (18) which reduces MHC II expression on B cells (19-22) but enhances MHC II expression on DC (23). Our data is therefore consistent with high-dose UVA protecting from immunosuppression via the release of IFNγ which in turn inactivates suppressor B cells. Thus high dose UVA-induced IFNγ may alter the balance of APC in draining lymph nodes, favouring DC. This intriguing possibility warrants further investigation as it may provide information on a key mechanistic intervention step that could ultimately

lead to the development of strategies aimed at enhancing systemic immunity and protecting from sunlight-induced immunosuppression.

One of the more striking effects of ssUV-irradiation we observed in our original study was the consistent and selective up-regulation of MHC II antigens on the surface of lymph node B cells (8). An increase in the expression of MHC II on B cells has traditionally been associated with an activated cell phenotype, one which may bias the immune response towards tolerance rather than immunity (24). Indeed, we have recently established that when these solar-simulated-activated lymph node B cells are co-injected with antigen loaded DC, they suppress the induction of immunity *in vivo* (8). From our current study it is clear that the UVB but not the UVA portion of the solar spectrum is responsible for this lymph node B cell activation. Hence, our data demonstrates that the early cellular target of immunosuppressive and tolerogenic UVB are lymph node B cells. Moreover it is clear that the mechanism of UVA-induced immunosuppression involves cellular targets other than lymph node B cells.

In conclusion, we have shown that immunosuppressive UVB, but not immunosuppressive UVA doses of the solar spectrum activate B cells in the draining lymph nodes. This is an important discovery because it has recently been demonstrated that these UV-activated B cells possess a suppressor phenotype *in vivo*. In contrast, not only did the UVA portion of sunlight fail to activate lymph node B cells, it suppressed B cell activation at higher doses which protect from UVB-induced immunosuppression. The findings have important implications for the development of strategies aimed at reducing the incidence of skin cancer and other UV-induced disorders.

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FIGURE CAPTIONS

Figure 1: The UVA and UVB spectra used in this study. The specra of the UVB (solid line) and UVA (dashed line) sources used in this study were measured with an Optronics spectroradiometer. The absolute irradiace of the UVB, UVA, and visible wavebands are shown in the insert.

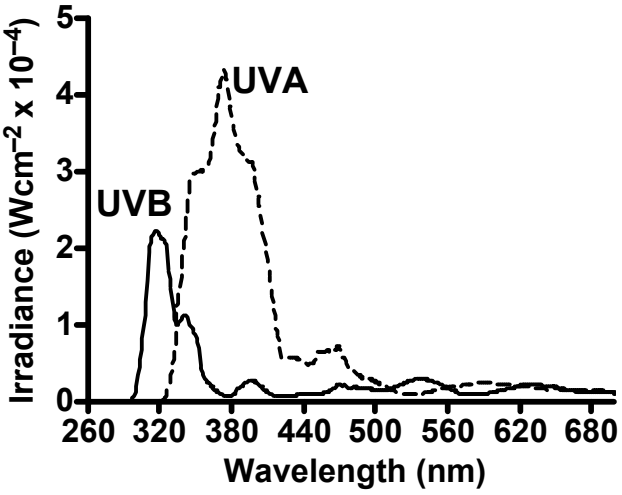
Figure 2: Both UVA and UVB suppress Primary Immunity, but only UVB induces Tolerance. The ability of UVA (n = 18 - 21 mice per dose) and UVB (n = 15 - 18 mice per dose) to suppress primary immunity (left panel) was assessed by sensitization 3 days after the last UV irradiation. Mice were then rested and the ability of UVA (n = 19 mice per dose) and UVB (n = 10 - 12 mice per dose) to suppress the secondary immune response was assessed 4 weeks later (right panel). Mean results from 4 separate normalised and pooled experiments ± SEM are shown. The absolute change in ear thickness was $29.7 \pm 1.9\ mm^{-2}$ for un-irradiated, sensitised and challenged control groups and $9.9 \pm 1.0\ mm^{-2}$ for unsensitised, challenged only irritant control groups. The irritant control values within each experiment has been subtracted from each treatment group. A two-tailed Student's t-test compared the CS from mice receiving UVB (*) or UVA (†) to the un-irradiated control groups, $p < 0.05$.

Figure 3: Exposure to UVB but not UVA increases total lymph node, B cell and dendritic cell numbers. The two inguinal skin-draining lymph nodes were removed from C57BL/6 mice 3 days after the last UV irradiation. Total lymph node cell number was determined using a haemocytometer and trypan blue to exclude dead cells. Lymph node cells were stained with specific antibodies and analysed by flow cytometry with B cells being identified as $CD11c^{-}MHC\ II^{+}B220^{+}IgM^{+}$ and DC as $CD11c^{+}MHC\ II^{+}IgM^{-}$. The percentage of B cells or DC was determined using CellQuest analysis software and total cell numbers were calculated by multiplying this percentage by the total number of lymph node cells. Mean results from 3 separate normalised and pooled experiments ± SEM are shown

(n = 9 for all doses). In one series of 3 pooled experiments, the absolute number of cells per draining lymph node was $5.0 \pm 0.3 \times 10^6$ in positive control groups and $9.1 \pm 0.6 \times 10^6$ in mice that received the highest dose of UVB. In these same groups, the absolute number of lymph node B cells was $0.8 \pm 0.2 \times 10^6$ and $2.0 \pm 0.5 \times 10^6$ cells while the absolute number of lymph node DC was $0.3 \pm 0.04 \times 10^6$ and $0.5 \pm 0.08 \times 10^6$ cells respectively. * $p < 0.05$ by a two-tailed Student's t-test comparing the cell numbers from mice receiving UV to the un-irradiated control groups.

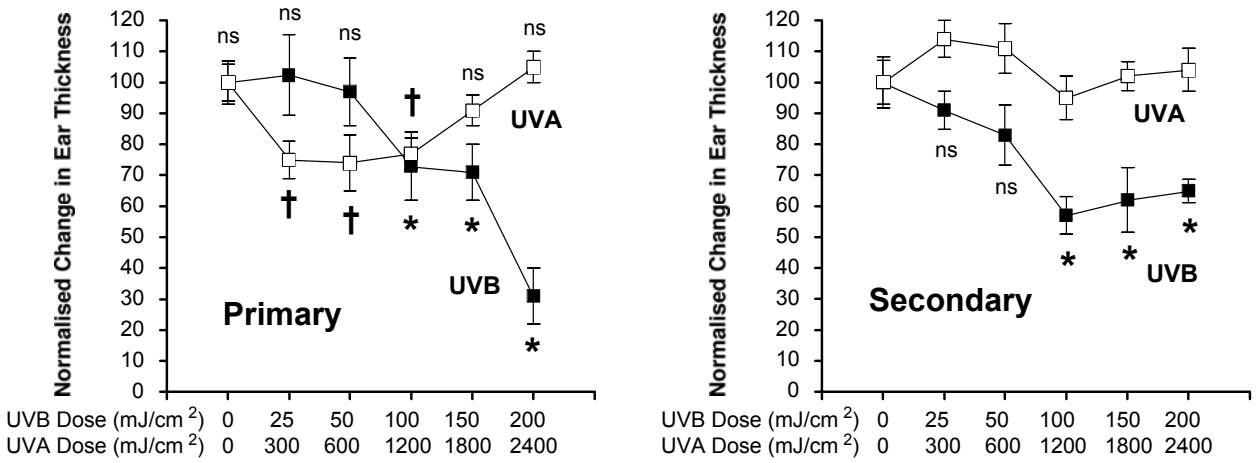
Figure 4: Immunosuppressive UVB Activates Lymph Node B cells While Immunoprotective UVA Activates Lymph Node DC. Inguinal lymph node cell suspensions were prepared and analysed by flow cytometry to determine the level of MHC II expression on the surface of DC ($CD11c^+MHC\ II^+$) and B cells ($CD11c^-MHC\ II^+B220^+$). For groups receiving UVB irradiation, mean results from 3 separate normalised and pooled experiments ± SEM are shown (n = 9). For groups receiving UVA irradiation mean results from 5 separate normalised and pooled experiments ± SEM are shown (n = 15). * $p < 0.05$ by a two-tailed Student's t-test comparing MHC II expression to the un-irradiated control groups.

Byrne et al. – Figure 1

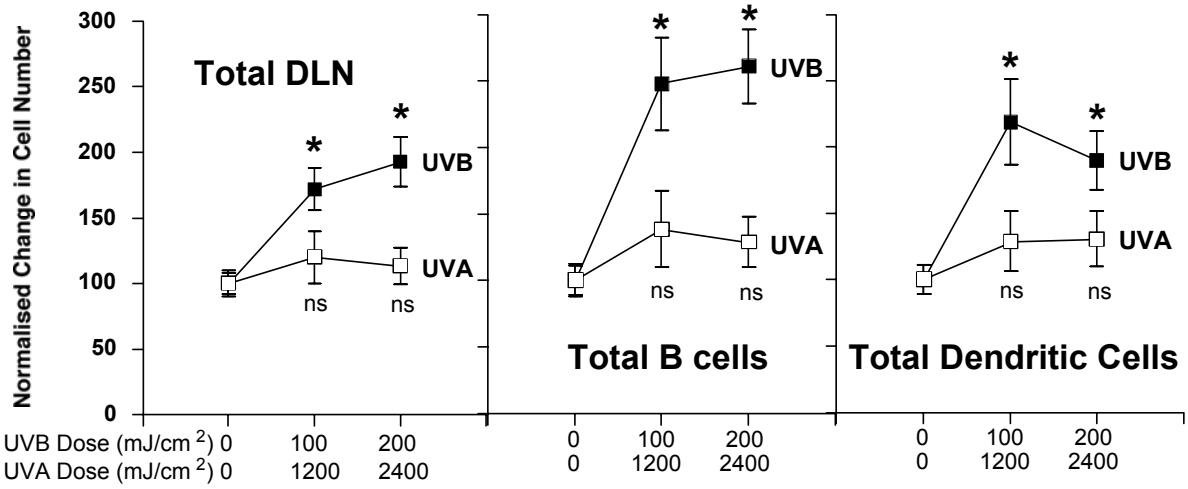


Spectra	Irradiance (Wcm^{-2}) 290-320nm	320-400nm	400-700nm
UVA	4.30×10^{-7}	2.12×10^{-2}	1.12×10^{-2}
UVB	2.98×10^{-3}	5.20×10^{-3}	4.95×10^{-3}

Byrne et al. – Figure 2



Byrne et al. – Figure 3



Byrne et al.- Figure 4