

B Cells Activated in Lymph Nodes in Response to Ultraviolet Irradiation or by Interleukin-10 Inhibit Dendritic Cell Induction of Immunity

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Ultraviolet (UV) radiation suppresses systemic immunity. We explored these cellular mechanisms by exposing mice to systemically immunosuppressive doses of UV radiation and then analyzing cell phenotype and function in the lymphoid organs. Although UV radiation increased total cell number in the draining lymph nodes (DLN), it did not alter the activation state of dendritic cells (DC). Rather, UV radiation selectively activated lymph node B cells, with these cells being larger and expressing higher levels of both anti-major histocompatibility complex II and B220 but not co-stimulatory molecules. This phenotype resembled that of a B cell geared toward immune tolerance. To test whether UV radiation-activated B cells were responsible for immunosuppression, DC and B cells were conjugated to antigen *ex vivo* and transferred into naïve hosts. Although DC by themselves activated T cells, when the B cells from UV radiation-irradiated mice were co-injected with DC, they suppressed DC activation of immunity. Interleukin (IL)-10-activated B cells also suppressed DC induction of immunity, suggesting that IL-10 may be involved in this suppressive effect of UV radiation. These results demonstrate a new mechanism of UV radiation immunosuppression whereby UV radiation activates B cells in the skin-DLN that can suppress DC activation of T cell-mediated immunity.

Key words: B lymphocytes/dendritic cells/immune suppression/interleukin-10/sunlight
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The ultraviolet (UV) wavelengths in sunlight are a major human health concern because they cause both DNA damage and immunosuppression, both of which are required for the development of skin cancers (Kripke, 1994). UV radiation is divided into three wavebands, UVC (<290 nm), UVB (290–320 nm), and UVA (320–400 nm), with visible light being made up of even longer wavelengths. UVC is blocked by the ozone layer and does not reach the Earth's surface so sunlight is a mixture of UVB and UVA.

UV suppresses the induction of local and systemic immune responses as well as reactivation of immunological memory (Damian *et al*, 1999; Byrne *et al*, 2002). UV-induced "local immunosuppression" is observed when an area of skin (e.g., dorsum) is irradiated followed by application of antigen to the same skin site. This paper has studied systemic immunosuppression that is observed when one site receives the UV (e.g., dorsum) but the antigen is applied to a site that did not receive the UV (e.g., abdomen). This process is mediated via a cascade release of cytokines including interleukin (IL)-4 and IL-10 that culminate in the activation of

suppressor cells in secondary lymphoid tissues (Shreedhar *et al*, 1998; Moodycliffe *et al*, 2000). The cellular events involved in the activation of these suppressor cells, however, remain unclear. It is known that UVB can suppress the production of IL-12 by lymph node dendritic cells (DC) (Kitazawa and Streilein, 2000; Schmitt and Ullrich, 2000), which is likely to be an important event because DC can activate effector-, and in certain circumstances, regulatory T cells (Martin *et al*, 2003).

B cells are an important lymph node antigen-presenting cell (APC) normally responsible for activating secondary immune responses and producing antibody. But recent evidence also implicates B cells in the *regulation* of immune responses. IL-10-secreting B cells were found to regulate chronic intestinal inflammation (Mizoguchi *et al*, 2002) as well as temper the severity of autoimmune disorders (Fillatreau *et al*, 2002; Mauri *et al*, 2003). More importantly, there is now increasing evidence that B cell-dependent immune regulation may be mediated via inhibition of DC function (Moulin *et al*, 2000). In light of this intriguing possibility, we have investigated whether immunosuppressive doses of solar-simulated UV (ssUV) radiation alters the activation state of DC subsets and B cells in draining lymph nodes (DLN), and whether this alteration is responsible for inducing a state of immunological inactivity. We show for the first time that UV radiation activates B cells in the DLN and that these cells inhibit the induction of T cell immunity by DC.

Abbreviations: APC, antigen-presenting cell; CS, contact sensitivity; DLN, draining lymph node; FCS, fetal calf serum; HBSS, Hanks-buffered salt solution; IL, interleukin; LC, Langerhans cells; MHC, anti-major histocompatibility complex; ssUV, solar simulated ultraviolet radiation; Th1, T-helper type 1; TNCB, tri-nitro-chloro-benzene; TNP, tri-nitro-phenol; UV, ultraviolet

Results

Immunosuppressive ssUV alters DLN but not splenic or thymic cell numbers We first verified that 18200 mJ per cm^2 of ssUV causes systemic immunosuppression (Fig 1). The contact sensitivity (CS) response to both tri-nitro-chloro-benzene (TNCB) and oxazolone was suppressed 39% and 53%, respectively, by ssUV, showing that UV immunosuppression was not dependent on a particular antigen.

Comparisons between lymphoid organs of unirradiated and ssUV-exposed mice showed that whereas there was no alteration in spleen or thymus weights or total cell numbers, there was a significant increase in DLN weights ($5.1 \pm 0.3 \times 10^{-3}$ vs $7.7 \pm 0.5 \times 10^{-3}$ g, respectively; $n=12$) and cellularity (Fig 2). This increase in DLN size and cell numbers was not because of differences in body weights between unirradiated control groups and UV-irradiated mice (19.6 ± 0.3 and 19.8 ± 0.4 g, respectively; $n=12$).

ssUV alters the APC in DLN No significant alteration in splenic or thymic APC subsets was found following ssUV (data not shown). We identified total DLN DC as $\text{CD11c}^+ \text{MHC II}^+$ (MHC, anti-major histocompatibility complex), whereas B cells were $\text{CD11c}^- \text{MHC II}^+ \text{B220}^+$ (Fig 3A

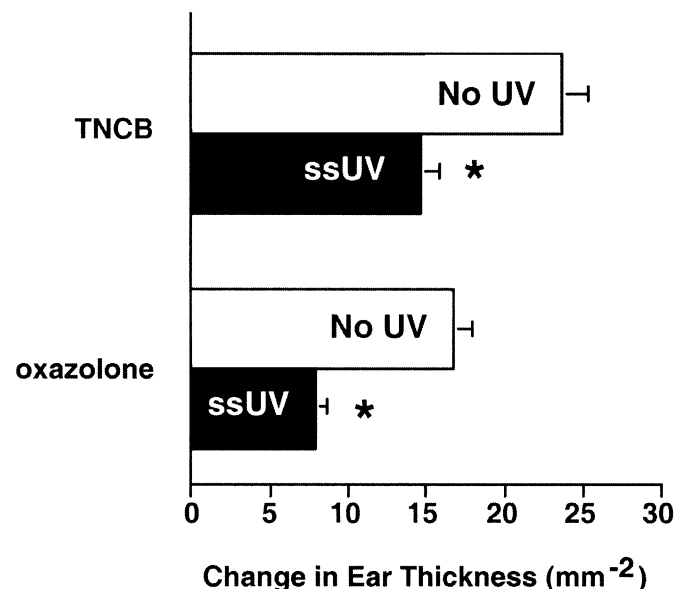


Figure 1
Eighteen thousand two hundred mJ per cm^2 of solar-simulated ultraviolet (ssUV) is immunosuppressive in C57BL/6 mice. Groups of five to six mice were shaved on their dorsal trunk and exposed to three consecutive daily doses of 18200 mJ per cm^2 ssUV, which were comprised of 1400 mJ per cm^2 UVB and 16800 mJ per cm^2 UVA. Three days following the final UV irradiation, the abdomens of both control unirradiated and UV-irradiated mice were shaved and the contact sensitizers tri-nitro-chloro-benzene (TNCB) or oxazolone were applied topically. Seven days later the right ear of each mouse (including an unsensitized irritant control group of mice) was challenged to the sensitizing antigen. The increase in ear thickness compared with the unchallenged left ear was measured 24 h later, and the response in irritant control mice was subtracted to assess immunity. Mean \pm SEM; $n=17$ for TNCB treatment (pool of three separate experiments), $n=5$ for oxazolone treatment. * $p < 0.05$ compared with unirradiated control.

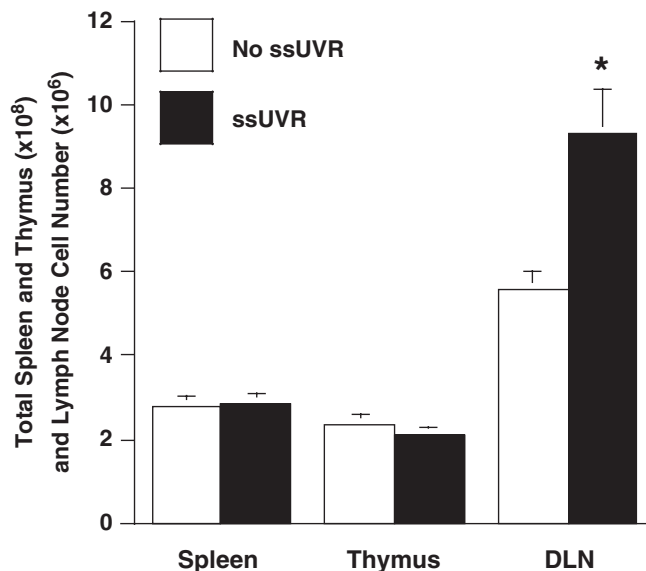
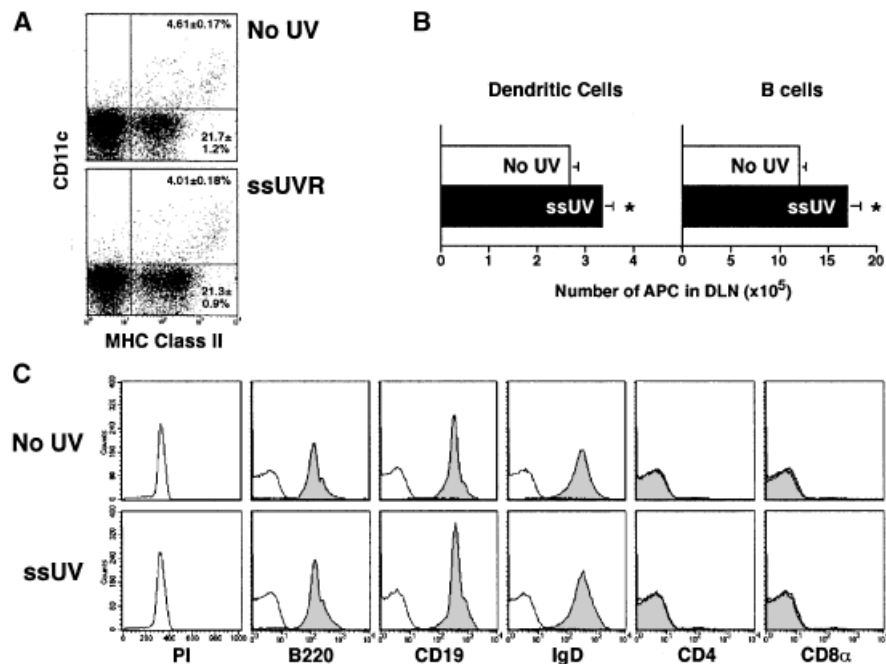


Figure 2
Solar-simulated ultraviolet (ssUV) does not affect spleen or thymus cell numbers, but significantly increases draining lymph node (DLN) cellularity. Groups of two mice were irradiated with ssUV or unirradiated. Three days after the last UV, corresponding exactly with the time that antigen would normally be applied to test for immune suppression, the spleen, thymus, and inguinal skin-draining lymph nodes were removed and made into cell suspensions and cell counts were performed. Mean \pm SEM of total cell counts for spleen ($\times 10^6$), thymus ($\times 10^6$), and DLN ($\times 10^6$) are shown for six repeats ($n=12$). * $p < 0.05$ compared with unirradiated controls.

and C). The total number of DC increased by almost 30%, whereas B cells increased by over 40% following exposure to ssUV (Fig 3B). This meant that ssUV increased the DLN ratio of B cells to DC from 4.7:1 to 5.5:1 ($p < 0.05$; $n=31$ and 32, respectively).

To confirm that we were correctly identifying B cells, DLN cell suspensions were labelled with other traditional B cell markers (Fig 3C). The $\text{CD11c}^- \text{MHC II}^+$ cells in the lower right quadrant of Fig 3A were also B220^+ , CD19^+ , and IgD^+ . This observation could not have been a result of doublet formation, with the presence of ethylenediamine-tetraacetic acid (EDTA) and PI staining verifying single cells. Additionally, these B cells were completely negative for the traditional T cell markers CD4 and CD8- α (Fig 3C). Further characterization to identify the subset of B cells found in the DLN of mice exposed to ssUV showed that whereas all B cells expressed high levels of both IgD and IgM (data not shown), they were almost completely negative for both CD5 and CD43 ($1.6 \pm 0.1\%$ and $1.8 \pm 0.1\%$ double positive for No UV and ssUV, respectively). Hence, B cells in the lymph nodes of both control and ssUV-irradiated mice are conventional B cells.

ssUV irradiation does not affect the number of phagocytic cells in the DLN We were interested in determining whether ssUV affects the ability of DLN cells to phagocytose particles. To test this, control and ssUV-irradiated mice were injected with fluorescent latex microspheres 18 h prior to the removal and analysis of DLN cells. Although ssUV reduced the total percentage of phagocytic cells in the DLN

**Figure 3**

Solar-simulated ultraviolet (ssUV) alters the number of antigen-presenting cells (APC) in the draining lymph node (DLN). (A) DLN single cell suspensions were labeled for CD11c and anti-major histocompatibility complex (MHC) II to identify the two major APC subsets (CD11c⁺MHC II⁺ dendritic cells (DC) and CD11c⁺MHC II⁻ B cells). The percentage of both DC (n=44 mice, pooled from 11 separate experiments) and B cells (n=32 mice, pooled from eight separate experiments) is shown in the upper right and lower right quadrants, respectively. (B) Total number of DC (n=44 mice, pooled from 11 separate experiments) and B cells (n=32 mice, pooled from eight separate experiments) in the DLN was calculated by multiplying the percentage of each APC by the total number of DLN cells for each mouse. Individual experiments contained groups of four mice either control unirradiated (open bars) or exposed to ssUV (solid bars). Mean ± SEM are shown; *p<0.05 compared with unirradiated control. (C) Three- and four-color flow cytometry confirmed the CD11c⁺MHC II⁺ B cells as B220⁺, CD19⁺, IgD⁺, CD4⁺, and CD8α⁻ (positive staining is shown by the shaded histograms and isotype staining is shown by the open histograms). PI staining verified that analysis was of single cells. Representative histograms are shown.

by 50% (Fig 4A), the absolute number of phagocytic cells in the DLN was unaffected by ssUV (Fig 4C). A detailed analysis of these cells showed that ssUV did not alter the ratio nor the number of B cells or DC that had phagocytosed beads *in vivo* (Fig 4B and C). Furthermore, B cells from either control or irradiated groups could both phagocytose particles *in vivo* (Fig 4B). The implications of these findings are 2-fold. First, they suggest that ssUV does not cause immunosuppression by altering the ability of lymph node APC to phagocytose antigen *in vivo*; and second, they show that the increase in cellularity that accompanies ssUV-induced immunosuppression is not because of the infiltration of the DLN by phagocytic cells.

ssUV alters the phenotype of B cells but not DC ssUV did not alter the distribution of DC subsets, or the phenotype of DC in the DLN compared with control groups. We identified the four main DC subsets in the DLN as CD11c^{low}B220⁺IgD⁻ (the so-called murine plasmacytoid DC that were also Gr-1⁺), CD8α⁺CD11c⁺, CD4⁺CD11c⁺, and CD11c⁺ DC, which were negative for all three of these lymphoid markers (Fig 5A). ssUV did not affect the subsets as a percentage of total DC (Fig 5B). Moreover, ssUV did not alter the activation state of DC in the DLN as the levels of MHC II and co-stimulatory molecules (CD80 and CD86) were unaffected by ssUV (Fig 5C). Thus, whereas ssUV increased the total number of DC in the DLN, this was not specific for any particular subset, and these DC were not activated.

Compared with CD11c⁺MHC II⁺ DC, DLN B cells expressed low levels of the co-stimulatory molecules CD80 and CD86 (Fig 6A). Similar to DC, this expression was not significantly altered by ssUV (Fig 6C). In contrast to DC, however, we found that ssUV significantly increased the expression of MHC II and B220 on DLN B cells (Fig 6B and C). Additionally, lymph node B cells were larger, as they had

increased forward and side scatter profiles (Fig 6B). These results show that ssUV does not alter the activation state of DC, but rather activates B cells in the DLN so that they become larger and express significantly higher levels of MHC II and B220.

DLN B cells activated by ssUV suppress DC initiation of T cell-mediated immune responses

To test whether the B cells activated by ssUV play a functional role in UV-induced immunosuppression, DLN DC and B cells from both control and ssUV-irradiated mice were purified with CD11c microbeads (Fig 7A), conjugated to antigen *ex vivo*, and injected into naïve syngeneic hosts to test their ability to activate immunity. Seven days later, the magnitude of the immune response induced by the antigen-coupled APC was tested by applying antigen (TNCB) to the surface of one ear and 24 h later measuring the increase in ear thickness. Whereas B cells from neither control nor ssUV-irradiated mice could activate effector T cells by themselves (Fig 7B, column 3), DC from both of these groups were able to induce significant levels of CS (Fig 7B, row 1). When the activated B cells from UV-irradiated DLN were combined with the DC from either control or UV-irradiated mice, however, they substantially inhibited the ability of these DC to activate immunity (Fig 7B, row 3). In contrast, B cells isolated from normal control mice did not inhibit immune induction when they were combined with DC (Fig 7B, row 2). It appears that there was a slight decrease in the level of immunity generated in mice that received DC from ssUV-irradiated mice together with B cells from unirradiated mice (Fig 7B, row 2, column 2). But this was not statistically different compared with groups that received ssUV DC only (Fig 7B, row 1, column 2; p=0.453), nor compared with groups that received a combination of No UV DC and No UV B cells (Fig 7B, row 2, column 1; p=0.2846). Moreover, there was a significant difference between groups that received ssUV DC only,

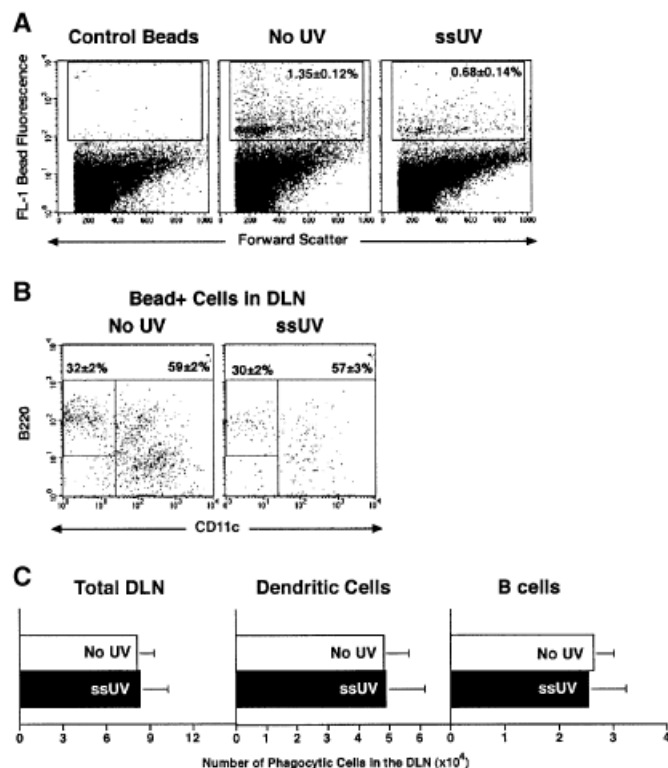


Figure 4
Solar-simulated ultraviolet (ssUV) irradiation does not affect the number of phagocytic cells in the draining lymph node (DLN). (A) Control mice ($n = 6$) or mice exposed to ssUV ($n = 5$) were injected with fluorescent latex beads i.v. 18 h prior to the removal of the DLN to analyze phagocytic ability of the antigen-presenting cells. Identical non-fluorescent negative control latex beads were injected into some mice to determine background fluorescence. Figures in the upper right corner refer to the absolute percentage of bead⁺ cells in the DLN. Representative dotplots shown. (B) Three- and four-color flow cytometry allowed for the detection of bead⁺CD11c⁺B220⁺ B cells and bead⁺CD11c⁺ dendritic cells (DC). Figures above the gates refer to the percentage of bead⁺ cells. (C) Total number of phagocytic cells and phagocytic DC or B cells was determined by multiplying the percentage by the total number of DLN cells. Results of two separate and pooled experiments are shown; * $p < 0.05$ compared with unirradiated control.

or ssUV DC together with No UV B cells and groups that received ssUV DC and ssUV B cells (Fig 7B, row 3, column 2; $p = 0.0338$ and 0.0366 , respectively). These comparisons support our theory that B cells from ssUV-irradiated mice but not normal B cells can suppress DC initiation of immunity. Similar results were obtained when these experiments were repeated using a different contact antigen (fluorescein isothiocyanate (FITC); results not presented). To verify that this B cell-mediated suppression of DC function was not a result of contaminating DLN T cells, we further purified B cells and T cells from ssUV-irradiated mice using CD90 microbeads (Fig 7C). The addition of T cells from ssUV-exposed mice did not affect the ability of DC to activate tri-nitro-phenol (TNP)-specific cells (Fig 7D, row 3). Furthermore, DC that were not incubated with the antigen *ex vivo* did not activate immunity (Fig 7D, row 2). These results show that DC from the DLN of ssUV-irradiated mice, in the absence of B cells, can function normally. More importantly, they demonstrate that UV-activated B cells (but not resting

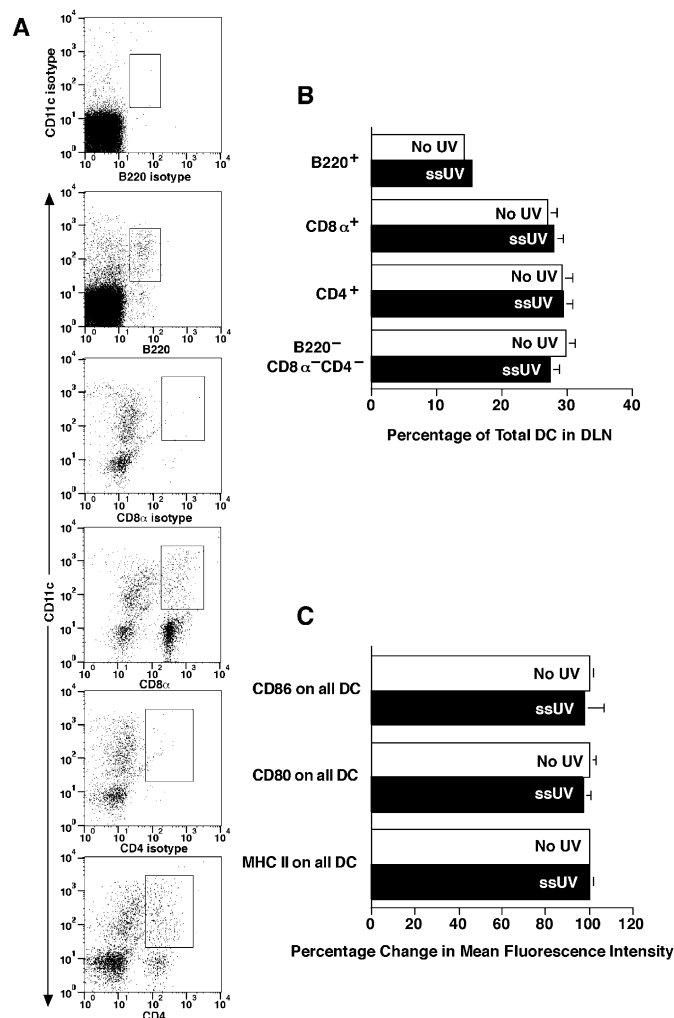


Figure 5
Solar-simulated ultraviolet (ssUV) does not alter the phenotype or subset distribution of draining lymph node dendritic cells (DLN DC). (A) DC subsets in the DLN were identified by three- and four-color flow cytometry first as CD11c⁺ anti-major histocompatibility complex (MHC) II⁺ and then using IgD-B220⁺ for plasmacytoid DC, CD8 α ⁺, CD4⁺ or negative for all three lymphoid markers. (B) ssUV did not alter the ratio of the various DC subsets in the DLN (mean \pm SEM are shown, $n = 12$ from a pool of six separate experiments). (C) ssUV did not alter the levels of MHC II or the co-stimulatory molecules CD80 or CD86 on total DC preparations (mean \pm SEM are shown, $n = 12$ from a pool of three separate experiments). Fluorescence intensities of cells from ssUV-irradiated mice were normalized to the mean fluorescent intensity of cells from control unirradiated mice. SEM not obvious are too small to be seen.

B cells from control mice or T cells from ssUV-exposed mice) inhibit DC-mediated activation of immunity.

To test whether IL-10 could induce a similar activated phenotype and suppressive function in B cells, freshly isolated naïve B cells were incubated for 24 h in IL-10 containing RPMI tissue culture media. Similar to ssUV irradiation, IL-10 upregulated both MHC II and B220 on B cells (Fig 8A). Moreover, when IL-10-activated B cells were co-injected with freshly isolated, TNP-conjugated DC, they were able to suppress the induction of immunity (Fig 8B). B cells by themselves did not activate immunity. Antigen on B cells was not required to suppress DC induction of

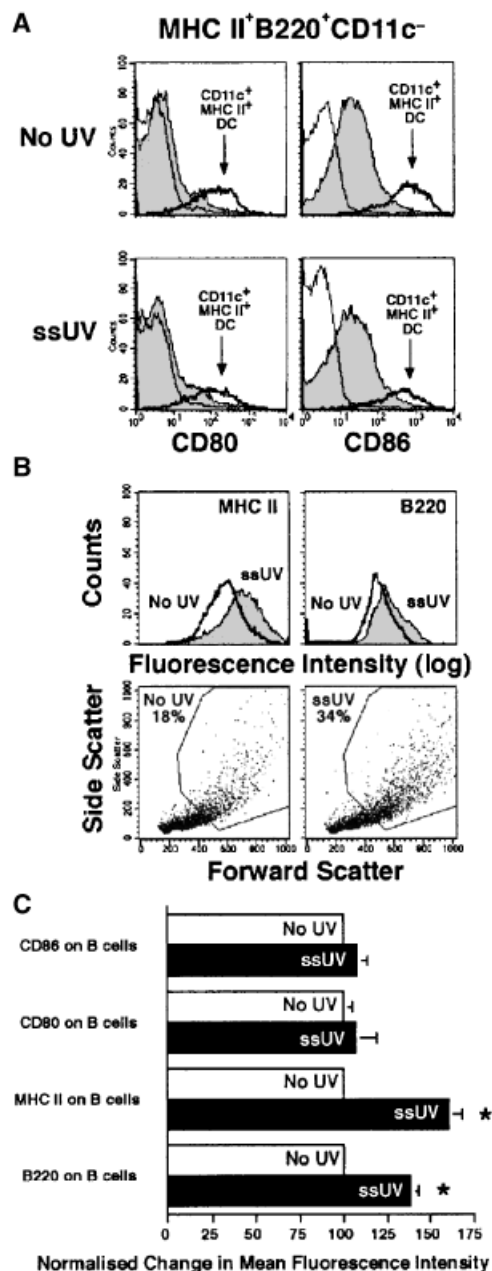


Figure 6

Solar-simulated ultraviolet (ssUV) activates draining lymph node (DLN) B cells. (A) Anti-major histocompatibility complex (MHC) II⁺B220⁺CD11c⁻ B cells were labelled by four-color flow cytometry for the co-stimulatory molecules CD80 and CD86. The levels of co-stimulatory molecule expression on DLN B cells (*shaded histogram*) compared with CD11c⁺MHC II⁺ dendritic cells (DC) (*bold line open histogram*) are shown for comparison. Isotype control staining on B cells is indicated by the thin-lined open histogram. (B) Change in expression of MHC II and B220 on B cells from control mice (*open histogram*) compared with ssUV-irradiated mice (*shaded histogram*). Forward versus side scatter profiles for B cells are shown in the two lower dotplots. The figure in the upper left corner is the percentage of B cells with a relatively high forward and side scatter profile. (C) ssUV did not alter the levels of co-stimulatory molecules CD80 or CD86 on DLN B cells (mean \pm SEM are shown, $n=12$ from a pool of three separate experiments). MHC II and B220 were significantly upregulated on DLN B cells from ssUV-irradiated mice ($n=32$, pooled from eight separate experiments). Mean \pm SEM are shown (SEM not obvious are too small to be seen); * $p<0.05$ compared with unirradiated control mice. Fluorescence intensities of cells from ssUV-irradiated mice were normalized to the mean fluorescent intensity of cells from control-unirradiated mice.

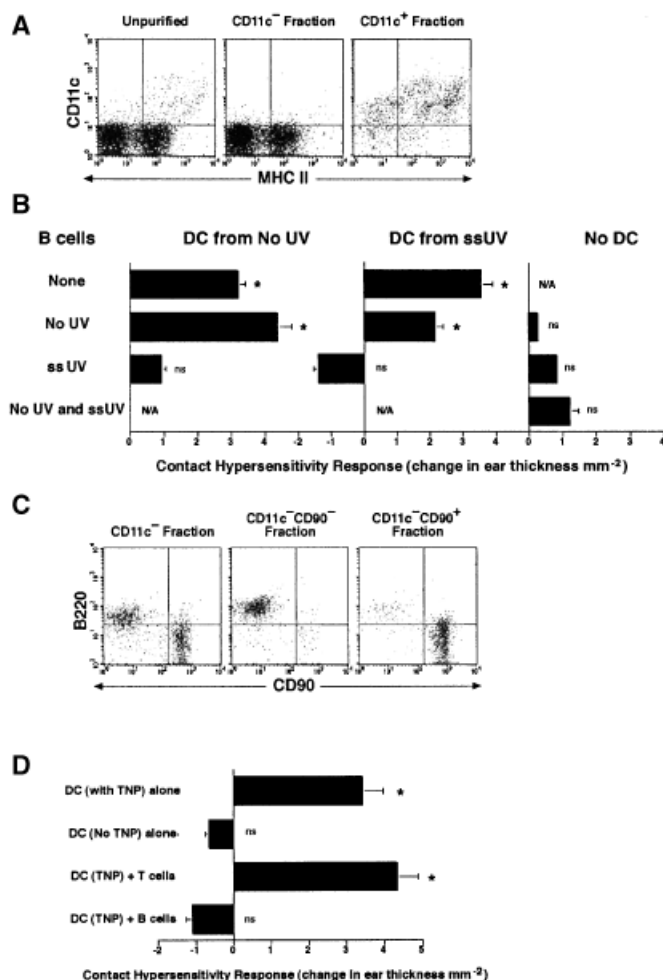


Figure 7

Solar-simulated ultraviolet (ssUV)-activated draining lymph node (DLN) B cells inhibit dendritic cells (DC) induction of T-helper type 1 (Th1) immunity. (A) DC and B cells from the DLN of both control unirradiated and ssUV-irradiated mice were purified using CD11c immunomagnetic beads. Typically, there was less than 0.25% contaminating DC in the B cell fractions and less than 3% contaminating anti-major histocompatibility complex (MHC) II⁺CD11c⁻ B cells in the DC fraction. (B) DC or B cells were then conjugated to tri-nitro-phenol (TNP) *in vitro* before 10^5 of each APC or a combination of each was injected i.v. into syngeneic naïve hosts. The top row shows the result when DC in the absence of B cells ($n=8$ for No UV and $n=18$ for ssUV donor DC) were injected into recipients. The second row shows the result when B cells from control unirradiated mice were injected either alone ($n=9$; *last column*), or in combination with control DC ($n=6$; *first column*) or DC from ssUV-irradiated mice ($n=9$; *middle column*). The third row shows the result when B cells from ssUV-irradiated mice were injected either alone ($n=8$; *last column*), in combination with control DC ($n=9$; *first column*), or DC from ssUV-irradiated mice ($n=10$; *middle column*). The fourth row shows a control showing the result when both B cell groups were co-injected ($n=6$). Results are a pool of three separate experiments. (C) DLN cells from ssUV-irradiated mice were purified for B cells and T cells by a two-step process using CD11c followed by CD90 microbeads. Both B cells and T cell fractions were greater than 95% pure. (D) DC, B cells, and T cells from ssUV-irradiated mice conjugated to TNP *ex vivo* or DC purified but not conjugated to TNP were then injected either alone (*rows 1 and 2* for DC; $n=9$ and 6) or in combination with T cells (*row 3*; $n=7$) or B cells (*row 4*; $n=7$). For all experiments, the mean change in ear thickness with irritant control groups subtracted \pm SEM is shown (irritant control absolute mean was 9.3 ± 0.9 per mm²; $n=20$). SEM not obvious are too small to be seen. * $p<0.05$ compared with the irritant control group, NS, not significant; N/A, not applicable. Representative dotplots from ssUV-irradiated mice are shown.

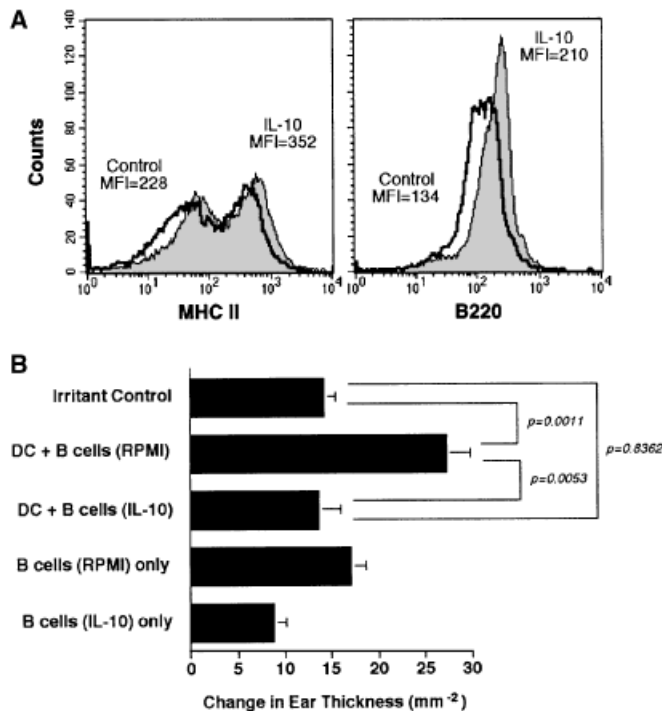


Figure 8
Interleukin (IL)-10-activated lymph node B cells inhibit dendritic cells (DC) induction of T helper type 1 immunity. (A) B cells were purified from naïve inguinal lymph nodes using CD11c and CD90 immunomagnetic beads similar to what is described for Fig 7C. Expression of anti-major histocompatibility complex (MHC) II and B220 is shown for B cells cultured in IL-10 for 24 h (closed histograms) or control RPMI only (open histograms). Figures refer to the mean fluorescence intensity. (B) DC were isolated from naïve inguinal lymph nodes, purified using CD11c immunomagnetic beads similar to what is described for Fig 7A, and conjugated to tri-nitro-phenol. DC were then co-injected i.v. with either IL-10 cultured (row 3; $n=5$) or RPMI-cultured (row 2; $n=5$) B cells. Some groups received IL-10 B cells (row 5; $n=6$) or RPMI B cells only (row 4; $n=5$). Irritant control groups are also shown (row 1; $n=6$). Mean \pm SEM are shown.

immunity, as this phenomenon was observed when B cells were either exposed to antigen (Fig 7B and D) or not exposed to antigen (Fig 8B). There was, however, an absolute requirement for antigen to be present on the DC for both immunosuppression and activation of immunity (Fig 7D). These results show that both ssUV and IL-10 activate lymph node B cells that are able to suppress DC induction of T cell immunity.

Discussion

UV radiation from the sun is one of the most damaging environmental carcinogens to which humans are exposed. In these experiments, we used a solar-simulated source of UV that provides an excellent reproduction of the UV portion of sunlight to demonstrate a new mechanism of how UV causes systemic immunosuppression. This process involves the activation of lymph node B cells that in turn suppress DC-mediated activation of T cell immunity.

Our data show that systemically immunosuppressive ssUV alters the cells in the lymph node draining UV-irradiated skin but not the spleen or thymus. ssUV clearly increased

the total DLN cell numbers, as well as the total number of all subsets of DC and B cells. A thorough phenotypic analysis of DLN B cells identified them as B220⁺MHC II⁺CD19⁺IgD⁺IgM⁺CD11c⁻CD4⁻CD8⁻CD5⁻CD43⁻. The increase in DLN cellularity is particularly interesting considering that this occurred in the absence of antigenic stimuli. The mechanism of how ssUV increases DLN cell numbers is unclear, but is almost certainly a prerequisite for the immune suppression that ensues. One possibility is that the increase in the number of DC and B cells was a result of migration of these cells from other organs. This hypothesis is supported by earlier experiments that showed that UVB induced changes in splenic adherent cells such that they activated suppressor T cells after trafficking from the spleen to the DLN (Greene *et al*, 1979; Gurish *et al*, 1982). Although we cannot rule out the possibility that ssUV has a similar effect on B cells and DC trafficking from the spleen to the DLN, we did not see any corresponding reduction in the number of spleen leukocytes. An alternative hypothesis is that the B cells migrated directly from the blood, lymph, or other lymph nodes as a consequence of ssUV induced "shut-down" of the lymph nodes.

In addition to the increase in cell numbers in the DLN, ssUV induced the selective activation of B cells, but not DC. We found that a significant proportion of the DLN B cells showed typical signs of cellular activation, with an increase in cell size, and upregulated expression of MHC II molecules (as well as B220). No concomitant increase in expression of the co-stimulatory molecules CD80 and CD86, however, was observed. This is potentially very important, as MHC II upregulation in the absence of adequate co-stimulatory molecule expression is thought to preference the induction of tolerance, rather than immunity (Croft *et al*, 1997). The ssUV-induced upregulation of B220 on B cells that we observed is particularly striking. B220 is one of the isoforms of CD45 created by alternate splicing. CD45 is a signal transduction molecule that regulates responses to antigen, integrin, and cytokine receptors, controlling the relative threshold of sensitivity to a variety of stimuli. It is a receptor-type protein tyrosine phosphatase involved in initiating immune responses in B and T cells (Hermiston *et al*, 2003). The role that B220 plays in ssUV-induced immune suppression, however, remains unclear. It is possible that ssUV initially triggers an increase in the systemic levels of both IL-4 and IL-10 (Shreedhar *et al*, 1998), which in turn increases the size and number, and upregulates the expression of MHC II and CD45 on B cells (Clark *et al*, 1989; Go *et al*, 1990). Indeed, this hypothesis is supported by our evidence that IL-10 activates B cells so that they have a phenotype similar to that of B cells isolated from ssUV-irradiated mice, and that these IL-10-activated B cells can also suppress DC induction of T cell immunity. The end result is to make lymph node B cells more susceptible to activation in response to antigen and thus contribute to the T-helper type 1 (Th1) to Th2 switch triggered by UV.

In addition to the obvious importance of these findings to skin carcinogenesis, ssUV-induced B cell activation may have consequences for the development of other tumors. Continual exposure to solar radiation may provide chronic activation signals to B cells. When this is combined with the immunosuppressive effects of such UV exposure, it is

perhaps not surprising that epidemiological studies have found an association between sunlight exposure and the development of non-Hodgkin's lymphoma, about 80% of which are B cell derived (Bentham, 1996; McMichael and Giles, 1996). Further supporting this hypothesis is the fact that UV irradiation of mice with only one functional copy of p53 increases the incidence of lymphoid malignancies, including B lymphocyte-derived tumors (Jiang *et al*, 2001).

Interestingly, in contrast to B cells, we did not observe any changes in the phenotype of DC in the DLN of ssUV-irradiated mice. Additionally, whereas the total number of DC increased, the actual percentage of DC with respect to all the other cells in the DLN actually decreased; thus, the ratio of DC to B cells decreased after ssUV. More importantly, there was no selective effect of ssUV on any one particular subset of DC. This means that ssUV does not cause systemic immunosuppression by altering DC phenotype or changing the balance of different DC subsets.

Exposing mice to ssUV had no effect on the ability of lymph node DC or B cells to phagocytose latex particles. We tested this by injecting control or irradiated mice with fluorescent latex beads 18 h prior to analysis. This technique has previously been used by us to successfully track phagocytic DC and macrophages infiltrating murine skin tumors (Byrne and Halliday, 2003). We found that exposing mice to ssUV had no effect on the number of phagocytic cells in the DLN. Although we did not directly analyze lymph node macrophages, it would appear from our data that phagocytic cells are not responsible for this UV-induced suppressive phenomenon. B cells from control and ssUV-irradiated mice were equally able to phagocytose the latex beads, as were DC from control and irradiated mice. Thus, we can rule out the possibility that ssUV suppresses immunity by altering the ability of B cells (or DC) to take up antigen.

UVB-induced immune suppression has long been associated with a switch from a Th1 cell mediated to a Th2-type humoral immune response (Ullrich, 1996). To test whether the B cells activated by ssUV were able to suppress Th1 immunity, we isolated the various APC subsets, conjugated them to antigen *ex vivo*, and used them to prime naïve mice. Interestingly, DC from both control and ssUV-irradiated groups, in the absence of activated B cells, were able to activate effector T cells in a typical Th1-mediated CS. This was somewhat surprising, because UVB has been shown to inhibit lymph node DC secretion of IL-12 (Kitazawa and Streilein, 2000; Schmitt and Ullrich, 2000). The mechanism of this altered DC function, however, has never been elucidated. We suspect that the activated B cells inhibit DC-initiated Th1 immunity by interfering with IL-12 secretion. This hypothesis is supported by numerous independent reports showing that B cell-deficient mice have increased DC-derived IL-12 (Moulin *et al*, 2000) and that whereas T cells potentiate IL-12 secretion by DC, B cells have the opposite effect (Rizzitelli *et al*, 2002). Indeed, it has only recently been shown that sustained IL-12 release is absolutely required for Th1 development (Athie-Morales *et al*, 2004). Any interference to this release of IL-12 could have major implications for the type of immune response that develops. To this end, we showed that when the ssUV-activated B cells were combined with the DC from either group, activation of the

Th1 response was inhibited. The mechanism for this regulatory activity by activated B cells is unclear, although there is now considerable evidence that regulatory and effector B cells do exist and that they can regulate immune responses via secretion of IL-10 (Harris *et al*, 2000; Fillatreau *et al*, 2002; Mizoguchi *et al*, 2002; Mauri *et al*, 2003). Therefore, our data now provide strong support for the hypothesis that ssUV (and the subsequent systemic release of IL-10) switches immunity from a Th1- to a Th2-type response by activating B cells that inhibit DC induction of cellular immunity. B cell-deficient mice do not mount a CS response (Tsuji *et al*, 2002) and therefore it cannot be tested whether these mice are resistant to systemic UV immunosuppression.

In conclusion, we have provided evidence for a novel mechanism of UV-induced immune suppression, one that involves the activation of B cells in the DLN that in turn regulate the initiation of the Th1 immune response via the inhibition of DC function. It suggests that ssUV "primes" the DLN for the arrival of antigen, such that if and when it arrives, the DLN is already set along the path of suppression rather than activation. In order to take advantage of this pathway for potential therapeutic intervention, it is necessary to explore the mechanisms of how these B cells become activated, and how they exert their regulatory activity on DC.

Materials and Methods

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

UV source The ssUV spectrum used in this study was produced with a 1000 W xenon arc solar simulator (Oriel, Stratford, Connecticut) as we have described previously (Byrne *et al*, 2002). The timing of UV exposure (in s) was adjusted with an automated timing device so that a dose of ssUV known to cause maximal immunosuppression could accurately be delivered to individual mice (Byrne *et al*, 2002).

UV irradiations For each experiment, groups of eight C57BL/6 mice had their back-skin hair removed using animal clippers (Oster, McMinnville, Tennessee) and then a close shave electric razor (Remington, Madison, Wisconsin) 24 h prior to irradiation. Two mice at a time were placed in a black Perspex animal-restraining device fitted with a quartz glass lid for exposure to the UV radiation. The ears and head were shielded from the UV with black Perspex. Irradiation times were about 30 s and a combination of fans and air-conditioning maintained body temperature during irradiation. Control unirradiated mice were shaved and restrained only. Mice were irradiated with 18200 mJ per cm² of ssUV (made up of 1400 mJ per cm² UVB and 16800 mJ per cm² UVA) each day for 3 consecutive days. Three days after the final UV irradiation, mice were either euthanized by cervical dislocation or contact sensitized.

Determination of immunosuppression by CS Mice that had received three consecutive doses of UV to the back-skin were sensitized to antigen by applying 50 μ L of a 4% wt/vol solution of TNCB (ICI Chemical, Tokyo, Japan) dissolved in 4:1 acetone:olive oil. To verify that the UV-induced immunosuppression occurred with another antigen, a separate group of mice was given 50 μ L of a 2% wt/vol solution of 4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone; Sigma Chemical, St Louis, Missouri) dissolved in acetone. These haptens were applied to the shaved unirradiated

abdomen 3 d after the final UV exposure, with positive control unirradiated mice being sensitized in the same way. To assess CS, 10 μ L of a 1% solution of TNCB in 4:1 acetone:olive oil or 5 μ L of the 2% oxazolone solution was applied to both sides of the right ear of the mice 7 d later. After a further 24 h, the difference in thickness between the right challenged and left unchallenged ears was measured using engineers callipers (Mitutoyo Corporation, Kanagawa, Japan). The increase in ear thickness of negative control unirradiated, unsensitized but challenged only mice (irritant control) were subtracted from the test groups.

Phagocytosis by lymph node APC *in vivo* Fifty four hours after the last UV irradiation, 200 μ L of Fluoresbrite Carboxy YG or control non-fluorescent 2.6% solid latex microspheres with a diameter of 0.5 μ m (Polysciences; Warrington, Pennsylvania) were injected i.v. into either UV-irradiated or control unirradiated mice. In these experiments, mice were not sensitized to antigen. Inguinal, back-skin DLN were then removed 18 h later (3 d after last UV) and made into single cell suspensions. The inguinal lymph nodes drain the site of UV irradiation (dorsum). Lymph nodes were teased apart with needles and then incubated in 1 mL of a 2% fetal calf serum (FCS)/Dulbecco's modified Eagle's medium (DMEM) (Trace Biosciences, Melbourne, Australia) solution containing 1 mg Collagenase II (Worthington Biochemical Corporation, Lakewood, New Jersey) and 0.1 mg DNase (Sigma Chemical). The cell suspension was periodically mixed at room temperature for 25 min before adding 85 μ L of 0.1 M EDTA (Sigma Chemical) for the final 5 min of the incubation and disaggregation. The cells were then strained through 70 μ m nylon gauze and centrifuged at $400 \times g$ through 400 μ L of FCS containing 10 mM EDTA. To ensure that only intracellular beads were assessed by flow cytometry, single cells were incubated with 1 mL of 20 mM EDTA in PBS for 20 min at room temperature prior to labelling with the antibodies described below.

Flow cytometry Each experiment contained groups of two to four control and two to four UV-irradiated animals. The left and right inguinal lymph nodes from individual mice were removed and pooled. In these experiments, mice were not sensitized to antigen. Single cell suspensions were prepared as described above and washed with 10% FCS/DMEM before pre-incubation at 4°C for 15 min with Fc-receptor blocking antibody (clone 2.4G2). In experiments analysing splenic and thymic cells, these organs were treated in the same manner. Cells were then placed in glass tubes for labelling with the following antibodies (clones) by four color flow cytometry; CD4 (RM4-5), CD5 (53-7.3), CD8 α (53-6.7), CD11c (HL3), CD19 (1D3), CD43 (S7), CD80 (16-10A1), CD86 (GL1), CD90 (30-H12), I-A^b (AF6-120.1), Gr-1 (RB6-8C5), IgD (11-26c.2a), IgM (II/41), and B220 (RA3-6B2) (all from Pharmingen, Franklin Lakes, New Jersey). Isotype control antibodies were used in parallel to ensure antibody specificity. Isotype control labelling was also used to produce the electronic gates for analysis of positively labelled cells. Streptavidin allophycocyanate (Pharmingen) was routinely used to label biotinylated primary antibodies. 10^6 cells in 100 μ L of primary or isotype control antibody at the same protein concentration (1–2 μ g antibody per 10^6 cells) were incubated for 45 min at 4°C. Cells were then washed by underlaying with 400 μ L of FCS and spun at $420 \times g$ before resuspending in 400 μ L of fresh DMEM/10% FCS for flow cytometry. Acquisition was performed on a FACSCalibur Flow Cytometer (Becton Dickinson, BD Franklin Lakes, New Jersey) and populations were analyzed using Cell Quest Pro software (BD).

Cell purification and adoptive transfer Draining inguinal lymph nodes pooled from either UV-irradiated or control unirradiated mice were made into single cell suspensions as described above. In these experiments, mice were not sensitized to antigen; the lymph nodes drain the site of UV exposure. CD11c and CD90 immunomagnetic beads were then used to purify CD11c⁺ DC, CD11c⁺CD90⁺ B cells, or CD11c⁺CD90⁺ T cells (MACS beads; Miltenyi Biotec, Auburn, California). Purified cells were conjugated

to the antigen TNP *in vitro* as we have described previously (Halliday *et al*, 1988). Briefly, cells were diluted to 10^7 cells per mL, an equal volume of 20 mM tri-nitro-benzene-sulfonic acid (ICI Chemical) in Hanks-buffered salt solution (HBSS) were added, and the cells were incubated for 10 min at 37°C. Cells were washed three times in 10% FCS/HBSS before resuspending in sterile HBSS. 10^5 APC and/or T cells (either DC or B cells by themselves or a co-transfer of cells in various combinations) in 200 μ L HBSS were then injected i.v. into naïve syngeneic hosts. Seven days after injection of cells, the immune response was elicited by challenging both sides of one ear with 10 μ L of a 1% TNCB solution and CS was measured 24 h later. The difference in thickness between the challenged and unchallenged ears was then read 24 h later in a blinded fashion. Irritant negative control mice were challenged with 10 μ L of 1% TNCB on each side of the ear but without prior sensitization. To verify that any changes were independent of the antigen used, a different contact antigen (FITC) was used in separate experiments. The method used to conjugate FITC to APC *in vitro* was similar to that above and has been described elsewhere (Knight *et al*, 1998).

IL-10 treatment of adoptively transferred B cells B cells were purified from the inguinal lymph nodes of unsensitized, unirradiated mice using CD11c and CD90 microbeads as described above. 2×10^5 purified B cells were then incubated in 96-well plates for 24 h with either complete RPMI/10% FCS media only (Invitrogen Corporation, Melbourne, VIC, Australia), or RPMI/10% FCS media containing 20 ng per mL of IL-10 (R&D Systems, Minneapolis, Minnesota) as described previously (Go *et al*, 1990). IL-10 was then removed by thoroughly washing the B cells four times with 10% FCS/HBSS. A sample of the B cells was stained for IgD, CD11c, B220, and MHC II to assess their activation state by flow cytometry. The remaining B cells were co-injected with freshly isolated, TNP-conjugated DC as described above. Seven days later, the immune response to TNP was measured by challenging the ears of mice with TNCB by a conventional contact hypersensitivity assay.

Statistical analysis For analysis of cell numbers, percentages and fluorescence intensities within lymphoid organs, an unpaired two-tailed Student's *t* test was used, where $p < 0.05$ was considered statistically significant. In the adoptive transfer experiments, immunity was defined as the ability of the APC to induce a significant CS to TNCB challenge above irritant negative control groups. Statistical analysis was carried out using an analysis of variance (ANOVA; Fisher's PLSD), with $p < 0.05$ considered statistically significant.

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References

- Athie-Morales V, Smits HH, Cantrell DA, Hilkens CMU: Sustained IL-12 signaling is required for Th1 development. *J Immunol* 172:61–69, 2004
- Bentham G: Association between incidence of non-Hodgkin's lymphoma and solar ultraviolet radiation in England and Wales. *Br Med J* 312:1128–1131, 1996

- Byrne SN, Halliday GM: Phagocytosis by dendritic cells rather than MHC II(high) macrophages is associated with skin tumour regression. *Int J Cancer* 106:736-744, 2003
- Byrne SN, Spinks N, Halliday GM: Ultraviolet-A irradiation of C57BL/6 mice suppresses systemic contact hypersensitivity or enhances secondary immunity depending on dose. *J Invest Dermatol* 119:858-864, 2002
- Clark EA, Shu GL, Luscher B, Draves KE, Banchereau J, Ledbetter JA, Valentine MA: Activation of human B cells. Comparison of the signal transduced by IL-4 to four different competence signals. *J Immunol* 143:3873-3880, 1989
- Croft M, Joseph SB, Miner KT: Partial activation of naive CD4 T cells and tolerance induction in response to peptide presented by resting B cells. *J Immunol* 159:3257-3265, 1997
- Damian DL, Barnetson RS, Halliday GM: Low-dose UVA and UVB have different time courses for suppression of contact hypersensitivity to a recall antigen in humans. *J Invest Dermatol* 112:939-944, 1999
- Fillatreau S, Sweeney CH, McGeachy MJ, Gray D, Anderton SM: B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* 3:944-950, 2002
- Go NF, Castle BE, Barrett R, *et al*: Interleukin 10, a novel B cell stimulatory factor: Unresponsiveness of X chromosome-linked immunodeficiency B cells. *J Exp Med* 172:1625-1631, 1990
- Greene MI, Sy MS, Kripke M, Benacerraf B: Impairment of antigen-presenting cell function by ultraviolet radiation. *Proc Natl Acad Sci USA* 76:6591-6595, 1979
- Gurish MF, Lynch DH, Daynes RA: Changes in antigen-presenting cell function in the spleen and lymph nodes of ultraviolet-irradiated mice. *Transplantation* 33:280-284, 1982
- Halliday GM, Cavanagh LL, Muller HK: Antigen presented in the local lymph node by cells from dimethylbenzanthracene-treated murine epidermis activates suppressor cells. *Cell Immunol* 117:289-302, 1988
- Harris DP, Haynes L, Sayles PC, *et al*: Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat Immunol* 1:475-482, 2000
- Hermiston ML, Xu Z, Weiss A: CD45: A critical regulator of signaling thresholds in immune cells. *Ann Rev Immunol* 21:107-137, 2003
- Jiang WD, Ananthaswamy HN, Muller HK, *et al*: UV irradiation augments lymphoid malignancies in mice with one functional copy of wild-type p53. *Proc Natl Acad Sci USA* 98:9790-9795, 2001
- Kitazawa T, Streilein JW: Studies on delayed systemic effects of ultraviolet B radiation on the induction of contact hypersensitivity. 3. Dendritic cells from secondary lymphoid organs are deficient in interleukin-12 production and capacity to promote activation and differentiation of T helper type 1 cells. *Immunology* 99:296-304, 2000
- Knight SC, Iqbal S, Roberts MS, Macatonia S, Bedford PA: Transfer of antigen between dendritic cells in the stimulation of primary T cell proliferation. *Eur J Immunol* 28:1636-1644, 1998
- Kripke ML: Ultraviolet radiation and immunology: Something new under the sun-presidential address. *Cancer Res* 54:6102-6105, 1994
- Martin E, O'Sullivan B, Low P, Thomas R: Antigen-specific suppression of a primed immune response by dendritic cells mediated by regulatory T cells secreting interleukin-10. *Immunity* 18:155-167, 2003
- Mauri C, Gray D, Mushtaq N, Londei M: Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med* 197:489-501, 2003
- McMichael AJ, Giles GG: Have increases in solar ultraviolet exposure contributed to the rise in incidence of non-hodgkin's lymphoma? *Br J Cancer* 73:945-950, 1996
- Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK: Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* 16:219-230, 2002
- Moodycliffe AM, Nghiem D, Clydesdale G, Ullrich SE: Immune suppression and skin cancer development: Regulation by NKT cells. *Nat Immunol* 1:521-525, 2000
- Moulin V, Andris F, Thielemans K, Maliszewski C, Urbain J, Moser M: B lymphocytes regulate dendritic cell (DC) function *in vivo*: Increased interleukin 12 production by DCs from B cell-deficient mice results in T helper cell type 1 deviation. *J Exp Med* 192:475-482, 2000
- Rizzitelli A, Berthier R, Collin V, Candeias SM, Marche PN: T lymphocytes potentiate murine dendritic cells to produce IL-12. *J Immunol* 169:4237-4245, 2002
- Schmitt DA, Ullrich SE: Exposure to ultraviolet radiation causes dendritic cells/macrophages to secrete immune-suppressive IL-12p40 homodimers. *J Immunol* 165:3162-3167, 2000
- Shreedhar V, Giese T, Sung VW, Ullrich SE: A cytokine cascade including prostaglandin E2, IL-4, and IL-10 is responsible for UV-induced systemic immune suppression. *J Immunol* 160:3783-3789, 1998
- Tsuji RF, Szczepanik M, Kawikova I, *et al*: B cell-dependent T cell responses: IgM antibodies are required to elicit contact sensitivity. *J Exp Med* 196:1277-1290, 2002
- Ullrich SE: Does exposure to UV radiation induce a shift to a Th-2-like immune reaction? *Photochem Photobiol* 64:254-258, 1996