

Regulatory B cells induced by ultraviolet B through toll-like receptor 4 signalling contribute to the suppression of contact hypersensitivity responses in mice

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Summary

Background. Ultraviolet (UV) B irradiation is known to suppress contact hypersensitivity (CHS) responses in mouse models by suppressing immune responses. However, the cellular mechanisms responsible for UVB-induced systemic suppression remain unclear. Regulatory B cells have been reported to play an inhibitory role during CHS. It is presently unknown whether regulatory B cells contribute to the effect of UVB phototherapy.

Objective. To investigate the inductive effect of UVB on regulatory B cells and the underlying mechanisms by using a CHS mouse model.

Methods. CHS was induced with oxazolone, and evaluated by histopathology, flow cytometry, and quantitative real-time polymerase chain reaction.

Result. We found that UVB irradiation induced regulatory B cell expansion and ameliorated CHS. UVB-induced regulatory B cells contribute to systemic immunosuppression by inhibiting the proliferation of T cells. Moreover, we determined that toll-like receptor (TLR) 4, the expression of which was upregulated in B cells after UVB exposure, played an essential role in the induction of regulatory B cells.

Conclusion. Our data identified regulatory B cells as regulators of UVB-induced immunosuppression in CHS, and suggest the importance of the UVB–TLR4 axis in the generation of regulatory B cells.

Key words: contact hypersensitivity responses; regulatory B cells; ultraviolet B (UVB).

Ultraviolet (UV) B (wavelength of 290–320 nm), representing 5% of the total UV radiation in sunlight, is one of the most vital environmental factors affecting humans (1, 2). In addition to DNA damage, UVB exposure triggers

a series of cellular changes and modulates immunosuppression (3, 4). The deleterious consequences of UVB exposure include skin cancer, exacerbation of infectious diseases, and promotion of premature skin ageing; conversely, UVB exposure is used for the treatment of autoimmune disease of the skin (5, 6).

UVB can inhibit contact hypersensitivity (CHS) responses by suppressing the induction of local immune responses and systemic immune responses (7, 8). UVB-induced local immunosuppression is mediated by promotion of Langerhans cell apoptosis or inhibition of Langerhans cell activation. UVB-induced systemic immunosuppression is mediated via a cascade release of immunosuppressive mediators and the activation of

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suppressor cells in secondary lymphoid tissues (1, 9). However, the cellular events involved in the activation of these suppressor cells remain unclear.

B cells mediate multiple immune responses by producing antibodies, presenting antigens, providing costimulatory signals, producing cytokines, and promoting T cell differentiation. However, in recent years, evidence has also implicated the regulatory role of B cells in immune responses. Interleukin (IL)-10-secreting B cells or regulatory B cells have been found to regulate experimental autoimmune encephalitis, experimental autoimmune hepatitis, collagen-induced arthritis, and inflammatory bowel disease (10, 11). More importantly, regulatory B cells have been shown to play important inhibitory roles in skin inflammation, for example by the demonstration of augmentation of psoriasis-like skin inflammation and CHS in B cell-deficient mice (12, 13).

However, whether regulatory B cells contribute to UVB-induced immunosuppression in skin inflammation has hitherto been unknown. To examine this intriguing hypothesis, we investigated whether immunosuppressive doses of UVB irradiation alter the regulatory function of B cells in lymphoid tissues, and whether this alteration is responsible for inducing a state of immunological inactivity. We show for the first time that regulatory B cells induced by UVB irradiation inhibit T cell proliferation and contribute to the UVB immunosuppressive effects in CHS responses.

Materials and Methods

Animals

Wild-type (WT) C57BL/6 mice were purchased from the Guangdong Medical Laboratory Animal Centre (Guangdong, China). Rag1^{-/-} mice and toll-like receptor (TLR) 4^{-/-} mice (C57BL/6 background) were obtained from the Model Animal Research Centre of Nanjing University (Nanjing, China). All mice were bred in the animal facility of Shenzhen PKU-HKUST Medical Centre (Shenzhen, China) under specific pathogen-free conditions, and used between the ages of 6 and 8 weeks. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals.

CHS responses

The mouse model of CHS involved induction with oxazolone (OXA) (Sigma-Aldrich, St Louis, MO, USA) as previously described (13, 14). In brief, mice were sensitized with 50 μ l of OXA 2% in acetone/alcohol (1:3) on the shaved abdomen on day 0. On day 5, sensitized mice were

topically challenged with 25 μ l of 1% OXA on the ear. Ear thickness was measured with an engineer's micrometer (Mitutoyo, Ka-wasaki, Japan) before OXA challenge and 48 h after OXA challenge.

UVB irradiation

The whole-body hair of mice was removed with a close-shave electric razor 24 h prior to UVB irradiation. The head and sensitization area were shielded from the UVB with black Perspex. Mice were irradiated with 1400 mJ/cm² UVB (Philips, Eindhoven, The Netherlands) each day for 5 consecutive days (7, 15).

Flow cytometry assay

Antibodies [anti-CD3, anti-CD19, anti-CD16/CD32, anti-CD45, anti-B220, anti-CD80, anti-CD86, anti-TLR4, anti-major histocompatibility complex (MHC) II, and anti-IL-10] (all from eBioscience, San Diego, CA, USA) were used for flow cytometry according to the manufacturer's instructions. For intracellular cytokine detection, cells were stimulated *in vitro* with lipopolysaccharide (LPS) (10 μ g/ml; Sigma-Aldrich) or CpG oligonucleotides (ODN2395, 5 μ g/ml; InvivoGen, San Diego, CA, USA), phorbol 12-myristate 13-acetate (50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) for 5 h in the presence of monensin (eBioscience) before being analysed by flow cytometry (13, 16).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from purified cells by the use of Trizol reagent (Sigma-Aldrich) according to the provided instructions. Complementary DNA was generated with a reverse transcription kit (Promega, Madison, WI, USA). Real-time PCR was performed with universal SYBR green supermix (Bio-Rad, Hercules, CA, USA). Fold change of target gene expression was calculated by using the comparative method for relative quantification by normalization to the internal control β -actin. The following primers were used: β -actin forward, 5'-CGGTTCCGATGCCCTGAGGCTCTT-3'; β -actin reverse, 5'-CGTCACACTTCATGATGGAATTGA-3'; TLR4 forward, 5'-ATGGCATGGCTTACACCACC-3'; and TLR4 reverse, 5'-GAGGCCAATTTTGTCTCCACA-3'.

Adoptive transfer of cells

Splenic cells were purified by magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity

of the isolated B cell or T cell subpopulation was found to be >95% as assessed by flow cytometry. After two washing steps with phosphate-buffered saline (PBS), T cells (1×10^7 cells/0.2 ml) or T and B cells [T cell/B cell ratio = 1:1, 2×10^7 cells/0.2 ml] were injected intravenously into each Rag1^{-/-} mouse 24 h before CHS induction.

Histological analysis of ear section

Mouse ears were harvested 48 h after OXA challenge; central strips of the ears were fixed in 10% formalin and embedded in paraffin. Sections 4 μ m in thickness were stained with haematoxylin and eosin for histopathological evaluation.

T cell proliferation assay

Purified T cells, which were found to be >95% pure as assessed by flow cytometry, were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. For the *in vitro* assay, T cells were stimulated with anti-CD3 (1 μ g/ml)/anti-CD28 (0.5 μ g/ml) (eBioscience) antibodies, and CFSE intensity was analysed 72 h later. For the *in vivo* assay, T cells (1×10^7) were transferred into Rag1^{-/-} mice. CFSE intensity was analysed 5 days later.

Statistical analysis

Data were analysed with GRAPHPAD PRISM software (version 5; GraphPad Software, La Jolla, CA, USA) and presented as means $\pm$ standard error of the mean. For comparison between two groups, Student's two-sample *t*-test was used to determine significant differences. Multiple group comparisons were performed with one-way ANOVA of variance followed by the Mann–Whitney *U*-test to compare two individual groups. *p*-Values of <0.05 were considered to be significant.

Results

Elevated IL-10 expression in B cells after UVB irradiation

We first assessed whether UVB played a role in the induction of regulatory B cells. All of the body hair of WT mice was shaved, and the mice were then exposed to UVB for 5 successive days. The proportion, number, activation state (the expression of MHCII, CD80 and CD86 on B cells) and IL-10 production of B cells were detected by flow cytometry 48 h after the final irradiation. In Fig. 1a, cells in each

panel were gated from CD45⁺ cells. In Fig. 1c, d, cells in each panel were gated from B220⁺ cells.

Comparisons between non-irradiated and UVB-irradiated mice showed that, in the blood and spleen, there were no significant alterations in the proportion, quantity and activation stage of B cells. In the draining (inguinal) lymph nodes, the proportion, absolute number and MHCII expression of B cells were increased in UVB-exposed mice (Fig. 1a–c). Thus, UVB irradiation did not affect the number, proportion and activation state of systemic B cells, but increased the number, proportion and MHCII expression of B cells from draining lymph nodes.

IL-10 expression is a vital element in the identification of regulatory B cells (17, 18). Whether UVB induces regulatory B cells was determined by assessment of IL-10 production by B cells after UVB irradiation. As compared with B cells from non-irradiated mice, the proportion of IL-10⁺ B cells among total B cells and the absolute number of IL-10⁺ B cells from UVB-exposed mice were significantly increased in the spleen, blood, or lymph nodes (Fig. 1d).

To date, a number of regulatory B cell subsets with different phenotypes have been identified (19). We next profiled IL-10⁺ B cells according to established methods (20). The results showed that IL-10⁺ B cells were distributed among all of the B cell subsets. Further analysis indicated that the proportion of IL-10⁺ B cells among immature B cells (T2 in blood; T2 and T2M2P in spleen) was much higher than that among mature B cells (Figure S1). To confirm the regulatory function of IL-10⁺ B cells, IL-10⁺ B cells and IL-10⁻ B cells from UVB-irradiated WT mice were isolated with a regulatory B cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions and a previous study (21). Then, B cells were co-cultured with CFSE-labelled T cells. The proliferation of T cells was assessed 72 h after anti-CD3 and anti-CD28 stimulation. As compared with IL-10⁻ B cells, IL-10⁺ B cells significantly inhibited T cell expansion *in vitro* (Figure S2). These results showed that UVB could induce systemic regulatory B cell expansion in mice.

The generation of regulatory B cells contributes to UVB-induced immunosuppression in CHS

We next attempted to explore the role of regulatory B cells in UVB-induced immunosuppression in CHS mice. All of the body hair of mice was shaved, and mice were then sensitized with OXA on the shaved abdomen on day 0. Mice, whose heads and sensitization areas were shielded, were irradiated with UVB for 5 days from day 1. On day 5, sensitized mice were challenged with OXA on their ears. The samples were harvested 48 h after challenge. In Fig. 2c, cells in each panel were gated from CD45⁺ cells.

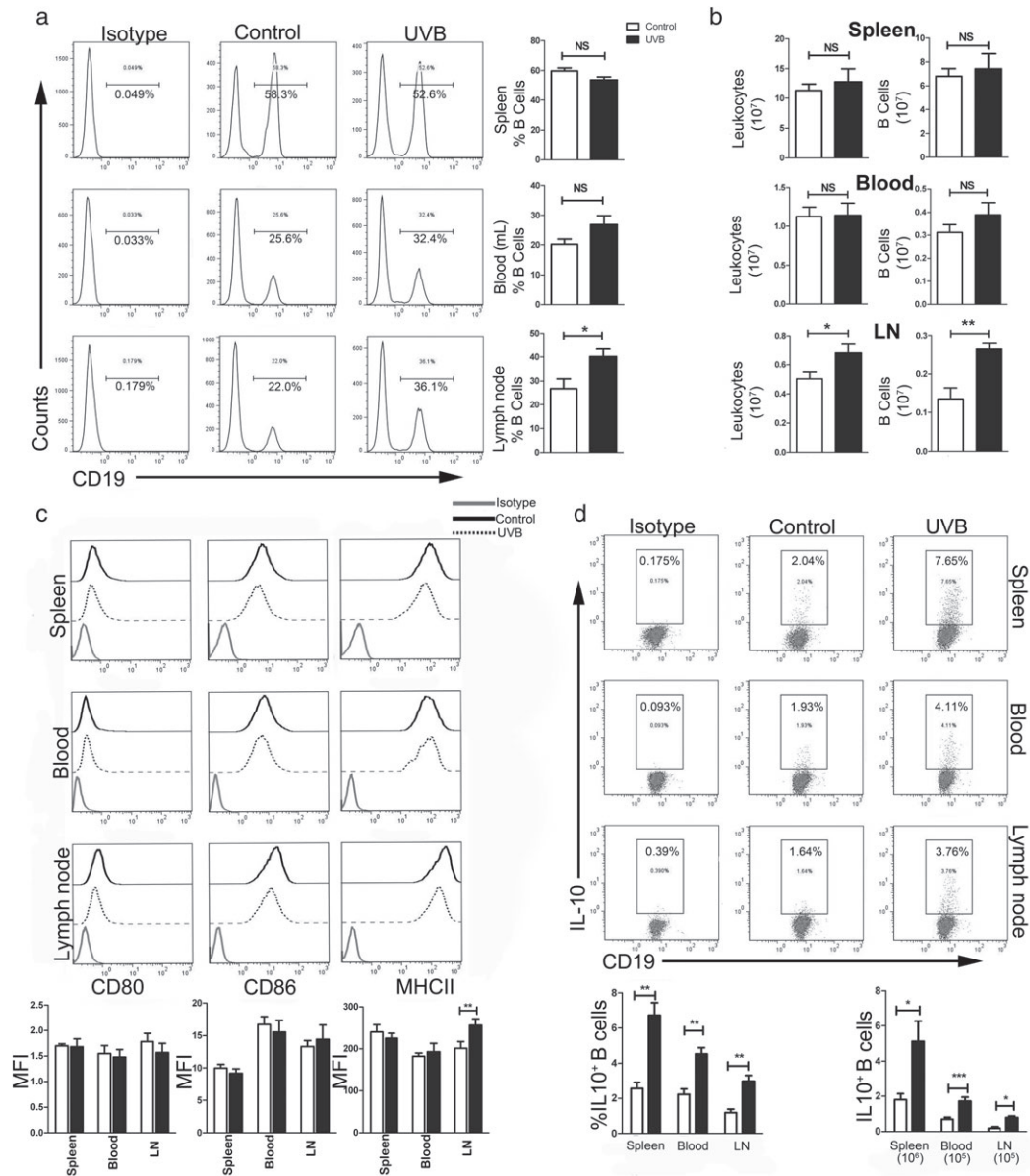


Fig. 1. Comparison of B cells between ultraviolet (UV) B-irradiated and non-irradiated mice. Cell suspensions of spleen, blood and lymph nodes (LNs) were prepared 48 h after final irradiation. **(a)** Percentages of CD19⁺ B cells in UVB-irradiated mice and non-irradiated mice. **(b)** Absolute numbers of leukocytes and B cells in UVB-irradiated and non-irradiated mice. **(c)** The expression of major histocompatibility complex II (MHCII), CD80 and CD86 on B cells from UVB-irradiated and non-irradiated mice. **(d)** The proportions and absolute numbers of interleukin (IL)-10⁺ B cells in UVB-irradiated and non-irradiated mice. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Data shown are the mean ± standard error of the mean from one experiment with 6 mice and three repetitions yielding similar results. MFI, mean fluorescence intensity; NS, not significant.

As compared with non-irradiated mice, UVB-irradiated mice had a significantly impaired CHS reaction (Fig. 2a, b). To investigate whether UVB modulates the expansion of regulatory B cells in CHS, the population and number of regulatory B cells were analysed by flow cytometry 48 h after challenge. In contrast to

the control non-irradiated CHS mice, UVB-irradiated CHS mice showed significant increases in regulatory B cells, both in proportion and in absolute number (Fig. 2c, d).

To confirm that the UVB-induced regulatory B cell expansion was attributable to UVB-induced immunosuppression in CHS, purified T cells with or without B cells

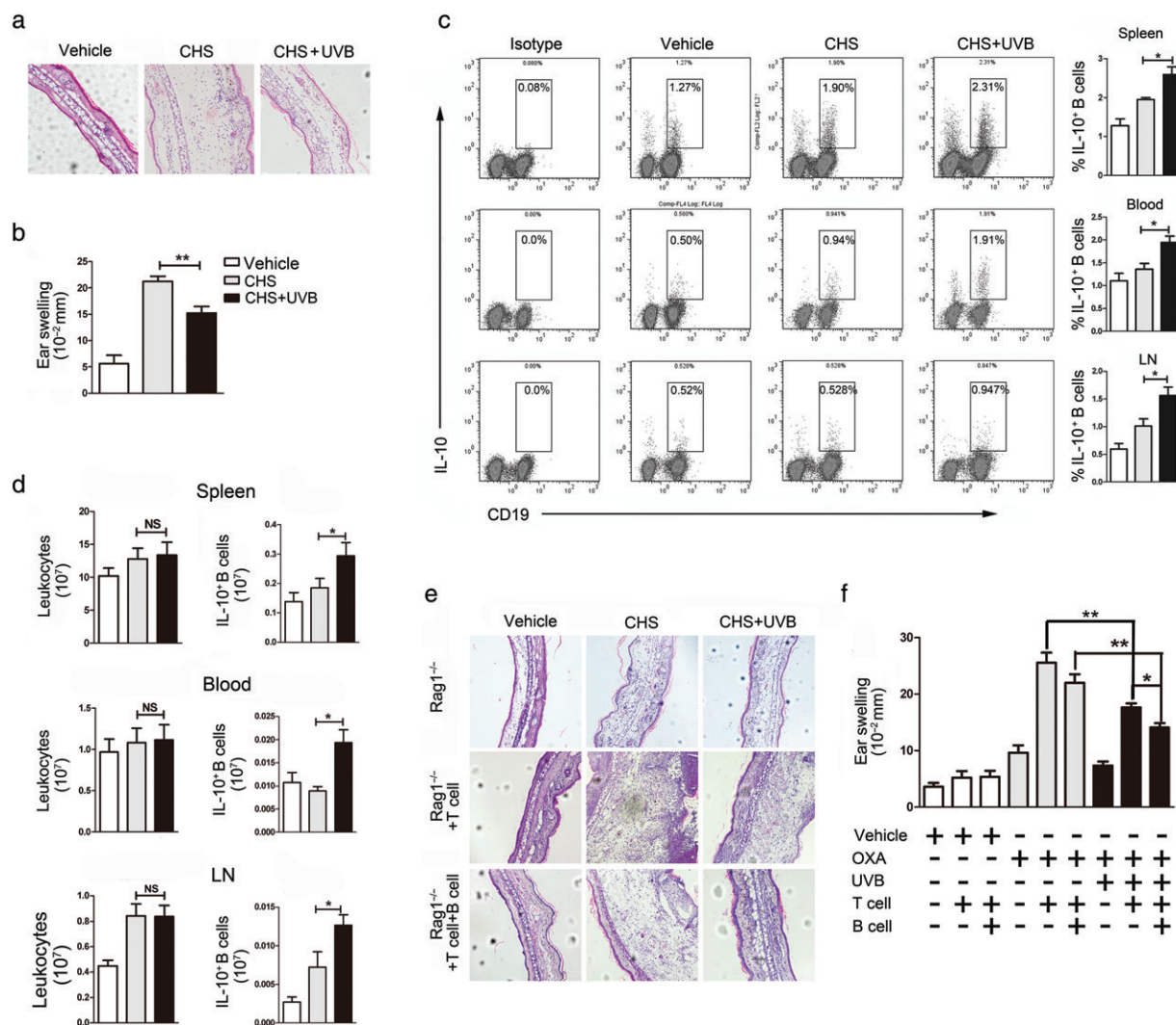


Fig. 2. Ultraviolet (UV) B induced regulatory B cell expansion and ameliorated contact hypersensitivity (CHS) in mice. **(a)** Representative ear sections were stained with haematoxylin and eosin, and examined at $\times 200$ magnification. **(b)** Ear swelling was measured with an engineer's micrometer. **(c)** The percentages of interleukin (IL)- 10^{+} B cells in UVB-irradiated and non-irradiated CHS mice. **(d)** The total numbers of leukocytes and IL- 10^{+} B cells in UVB-irradiated and non-irradiated CHS mice. **(e, f)** Rag1 $^{-/-}$ mice, Rag1 $^{-/-}$ mice that received T cells and Rag1 $^{-/-}$ mice that received T cells and B cells were treated with oxazolone (OXA) or OXA and UVB irradiation. Ear inflammation and ear swelling were assessed 48 h after OXA challenge. Data are pooled from three independent experiments, each with 6 mice, and are expressed as mean $\pm$ standard error of the mean. * $p < 0.05$, ** $p < 0.01$. NS, not significant.

from naive WT mice were adoptively transferred into Rag1^{-/-} recipient mice 24 h before CHS induction and UVB irradiation. Rag1^{-/-} mice that received T cells alone showed a severe CHS reaction that was attenuated by adoptive transfer of B cells. More importantly, the suppressive effect of UVB in CHS was significantly impaired in Rag1^{-/-} mice that received T cells alone (Fig. 2e, f). These data suggested that regulatory B cells that expanded after UVB irradiation participated in UVB-induced immunosuppression in CHS.

UVB-induced regulatory B cells restricted T cell proliferation both *in vivo* and *in vitro*

OXA-induced contact dermatitis is a T cell-mediated inflammatory response (22). To further confirm the regulatory function of UVB-induced regulatory B cells, B cells from UVB-irradiated mice were purified and co-cultured with CFSE-labelled T cells in different proportions. The proliferation of T cells was assessed 72 h after anti-CD3 and anti-CD28 stimulation. B cells from UVB-irradiated mice inhibited T cell proliferation in

a proportion-dependent way (Fig. 3a). Also, as compared with B cells from non-irradiated mice, B cells from UVB-irradiated mice significantly inhibited T cell expansion *in vitro* (Fig. 3b).

To investigate the regulatory function of UVB-induced regulatory B cells *in vivo*, T cells from CHS mice were purified and labelled with CFSE. B cells were purified from CHS mice that were non-irradiated or UVB-irradiated. Then, purified T cells alone or purified T cells with B cells either from UVB-irradiated or from non-irradiated CHS mice were adoptively transferred into Rag1^{-/-} recipient mice 24 h before OXA challenge. T cell expansion in the spleen, blood and lymph nodes was analysed 5 days after OXA challenge. As expected, T cells co-transferred with B cells from UVB-irradiated mice showed significantly decreased proliferation as compared with T cells transferred alone or co-transferred with B cells from UVB-non-irradiated mice (Fig. 3c). These data confirmed the regulatory function of B cells in UVB-irradiated mice, and suggested that UVB-induced regulatory B cells contributed to the therapeutic effect of UVB in CHS by inhibiting overexpansion of T cells.

UVB exposure increased the expression of TLR4 on B cells

TLR4 signalling in B cells plays an important role in the induction of regulatory B cells (23, 24). Recent studies also showed that TLR4 was required for UVB-induced immunosuppression (25). To explore whether TLR4 participates in the induction process of regulatory B cells, WT mice were exposed to UVB for 5 successive days. B cells from the spleen, blood and lymph nodes were harvested and purified 48 h after final irradiation. TLR4 expression on B cells was analysed by real-time PCR and flow cytometry. B cells from UVB-irradiated mice showed significantly higher TLR4 expression at the mRNA level than B cells from non-irradiated mice (Fig. 4a). As TLR4 is likely to be endocytosed after activation (26, 27), TLR4 expression at the protein level was analysed both extracellularly and intracellularly. Consistently, at the protein level, B cells from UVB-irradiated mice showed significantly higher expression of TLR4 than B cells from non-irradiated mice (Fig. 4b,c). These results suggested that increased TLR4 expression on B cells was an obvious consequence of UVB irradiation.

Decreased IL-10⁺ B cells in TLR4^{-/-} mice after UVB irradiation

To further investigate the role of TLR4 in the generation of regulatory B cells during UVB irradiation, WT mice and TLR4^{-/-} mice were exposed to UVB for 5 successive days.

The expression levels of active markers (CD80, CD86, and MHCII) and IL-10 were compared between WT B cells and TLR4^{-/-} B cells 48 h after final irradiation. The mean fluorescence intensities of active markers on B cells showed no significant difference between WT mice and TLR4^{-/-} mice (Fig. 5a). However, B cells from TLR4^{-/-} mice expressed a significantly lower level of IL-10 than B cells from WT mice after UVB irradiation (Fig. 5b). These data indicated that TLR4 participated in the generation of regulatory B cells during UVB irradiation.

B cells deficient in TLR4 impaired the suppressive effect of UVB on CHS

To further confirm whether TLR4 expressed on B cells was involved in UVB-induced immunosuppression in CHS, purified WT T cells with WT B cells or TLR4^{-/-} B cells were adoptively transferred into Rag1^{-/-} recipient mice 24 h before OXA sensitization. Mice receiving PBS or T cells alone were used as negative and positive controls, respectively.

OXA-sensitized mice were irradiated with UVB from day 1 to day 5. On day 5, sensitized mice were challenged with OXA on their ears. The severity of CHS was evaluated 48 h after OXA challenge. In the group treated with OXA and UVB, Rag1^{-/-} mice that received TLR4^{-/-} B cells showed increased swelling of the OXA-challenged ear as compared with Rag1^{-/-} mice that received WT B cells. However, in the group treated with OXA alone, no significant difference was observed between Rag1^{-/-} mice that received TLR4^{-/-} B cells and those that received WT B cells (Fig. 6a, b). The proportion of infiltrating T cells among total CD45⁺ inflammatory cells was analysed by flow cytometry 48 h after OXA challenge. Consistently, significantly decreased percentages of T cells were observed in Rag1^{-/-} mice that received WT B cells as compared with those that received TLR4^{-/-} B cells (Fig. 6c), indicating that regulatory B cells induced through the UVB–TLR4 pathway inhibited T cell infiltration in CHS mice. Together, these results showed that TLR4 signalling-induced regulatory B cells were triggered by UVB radiation and participated in the suppressive effect of UVB on CHS.

TLR4 deficiency impaired the inhibitory effect of B cells on T cells after UVB irradiation

To further confirm the inhibitory function of regulatory B cells generated through the UVB–TLR4 pathway, B cells from UVB-irradiated or non-irradiated WT mice and TLR4^{-/-} mice were isolated and co-cultured with CFSE-labelled T cells for 72 h in the presence of anti-CD3 and anti-CD28 stimulation. As expected, B cells from

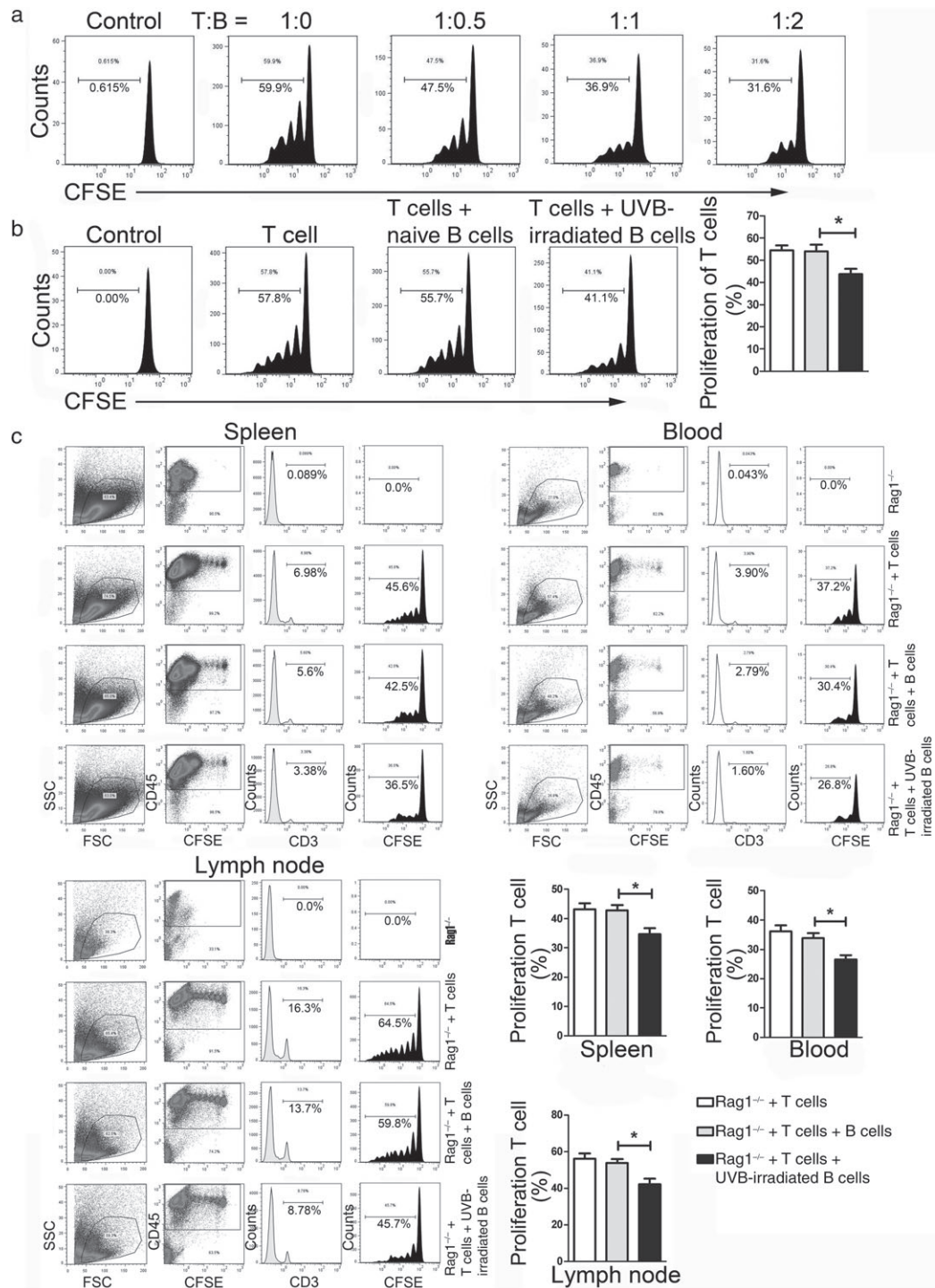


Fig. 3. Analysis of the regulatory function of B cells after ultraviolet (UV) B irradiation. **(a)** B cells were sorted from UVB-irradiated mice, and cultured with purified carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled T cells with different T cell/B cell ratios (T:B). **(b)** Purified CFSE-labelled T cells were cultured alone or with naive B cells or with B cells from UVB-irradiated mice (T:B = 1:1). **(c)** Purified T cells were sorted from oxazolone (OXA)-sensitized mice and labelled with CFSE. Purified B cells were sorted from UVB-irradiated or non-irradiated OXA-sensitized mice. Rag1^{-/-} mice that received T cells or T cells and B cells were challenged with OXA, and, 96 h later, the proliferation of T cells was analysed. Results are representative of three independent experiments with similar results, and are expressed as mean $\pm$ standard error of the mean. * $p < 0.05$.

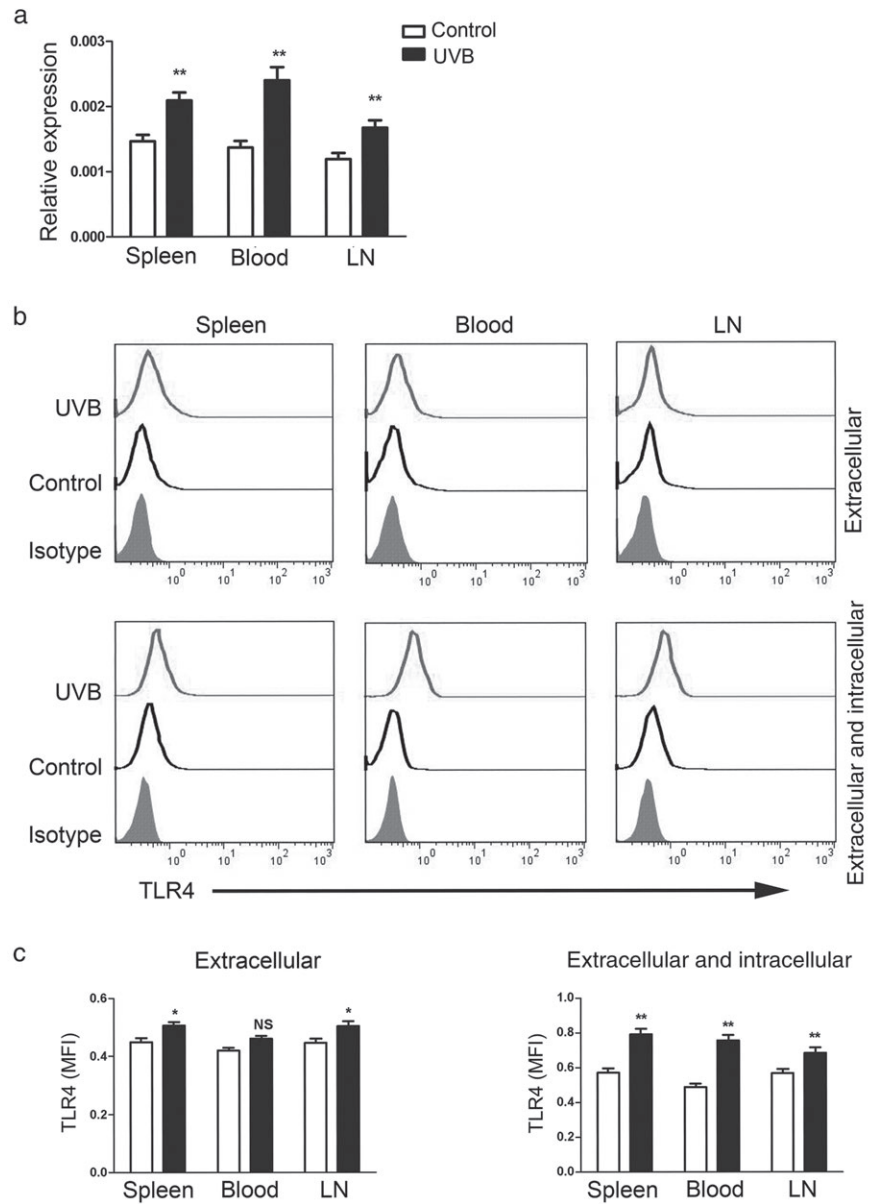


Fig. 4. The expression of toll-like receptor 4 (TLR4) after ultraviolet (UV) B irradiation. Mice were irradiated with UVB (1400 mJ/cm²) for 5 successive days. B cells from the spleen, blood and lymph nodes (LNs) were purified 48 h after final irradiation. **(a)** The expression of TLR4 on B cells was examined by quantitative real-time polymerase chain reaction. **(b)** TLR4 protein was examined extracellularly and intracellularly by flow cytometric assay. **(c)** (MFI of TLR4 protein is expressed as the mean $\pm$ standard error of the mean with $n = 6$). * $p < 0.05$, ** $p < 0.01$. MFI, mean fluorescence intensity; NS, not significant.

UVB-irradiated WT mice markedly inhibited T cell proliferation as compared with matched control B cells, but B cells from UVB-irradiated TLR4^{-/-} mice had no significant inhibitory effect as compared with matched control B cells (Fig. 7a).

To determine the effect of the UVB–TLR4 regulatory B cell axis in inhibiting antigen-specific T cell proliferation *in vivo*, we isolated OXA-primed T cells from OXA-sensitized mice, and B cells from WT mice and TLR4^{-/-} mice. Purified T cells alone or purified T cells with WT B cells or with TLR4^{-/-} B cells were adoptively transferred into Rag1^{-/-} mice. These mice were challenged with OXA and exposed to UVB for 5 successive

days. T cells from Rag1^{-/-} mice that received WT B cells showed low proliferative capacities as compared with those from Rag1^{-/-} mice that received TLR4^{-/-} B cells. Interestingly, on the other hand, WT B cells showed higher proliferative capacities than TLR4^{-/-} B cells (Fig. 7b). Collectively, these results demonstrate that the UVB–TLR4 regulatory B cell axis attenuates CHS by inhibiting T cell proliferation.

Discussion

Although UVB phototherapy is an effective option for treating inflammatory skin disease by inducing

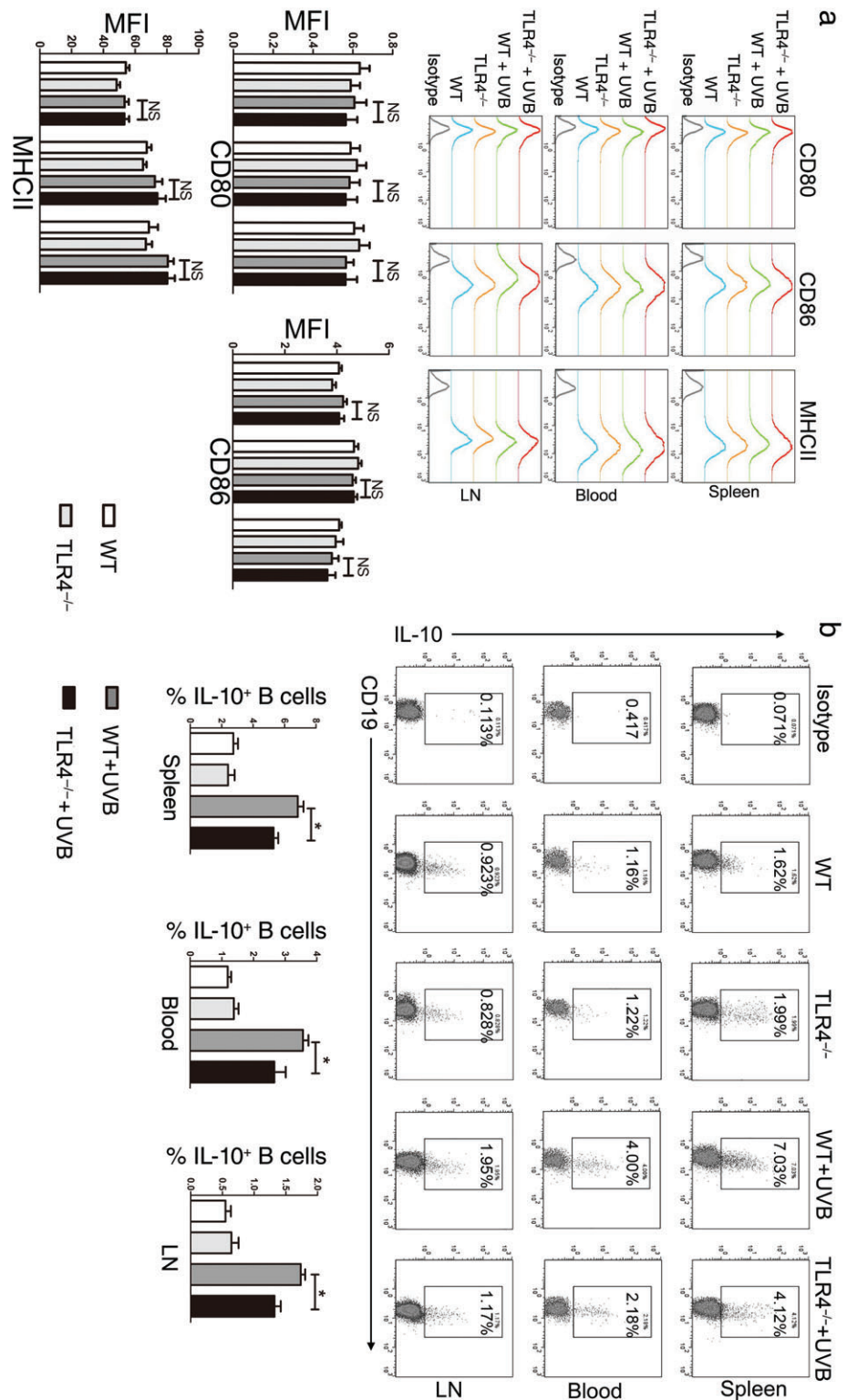


Fig. 5. The expression of activated markers and interleukin (IL)-10 in toll-like receptor 4 (TLR4)^{-/-} B cells and wild-type (WT) B cells after ultraviolet (UV) B irradiation. Mice were irradiated with UVB (1400 mJ/cm²) for 5 days. B cells from spleen, blood and lymph nodes (LNs) were prepared 48 h after final irradiation. **(a)** The expression of major histocompatibility complex II (MHCII), CD80 and CD86 on TLR4^{-/-} B cells and WT B cells from UVB-irradiated or non-irradiated mice. **(b)** The proportions of IL-10⁺ B cells among TLR4^{-/-} B cells and WT B cells from UVB-irradiated or non-irradiated mice. Data are pooled from three independent experiments, each with 6 mice, and are expressed as mean ± standard error of the mean (SEM). **p* < 0.05. MFI, mean fluorescence intensity; NS, not significant.

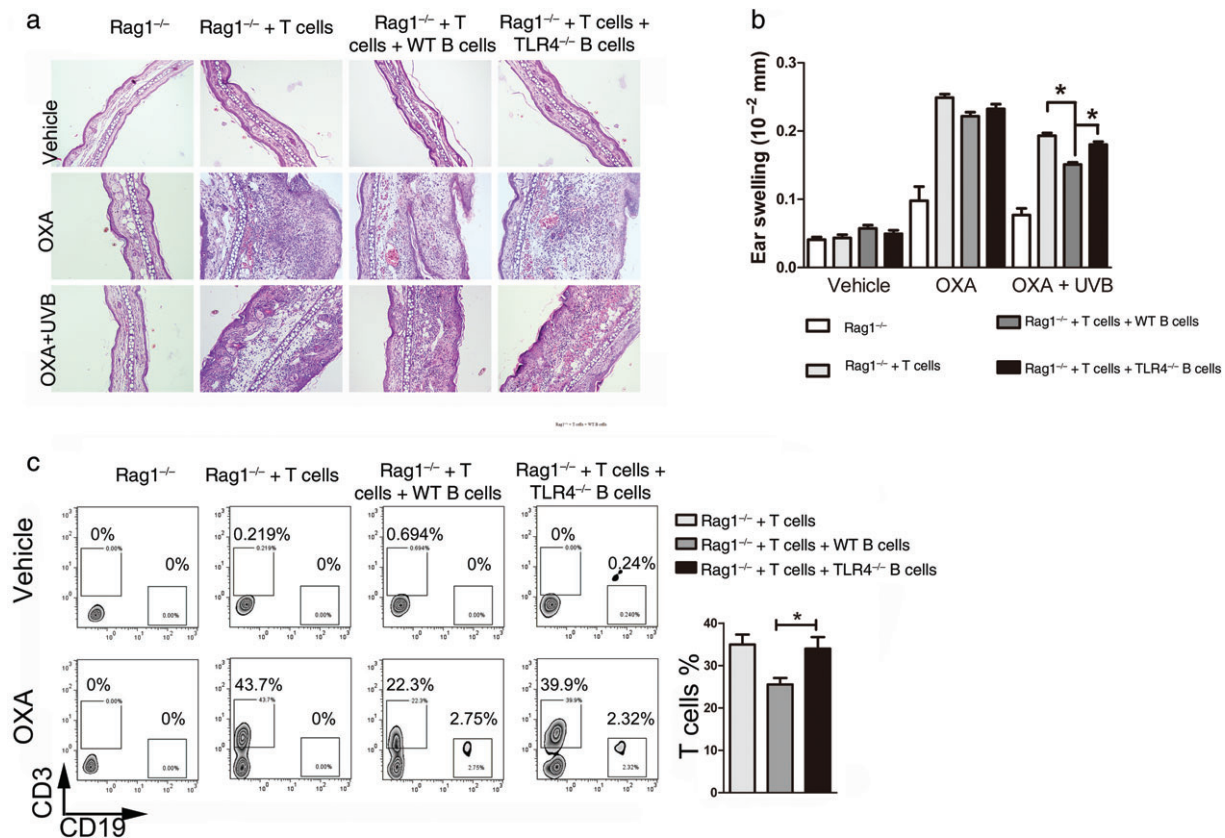


Fig. 6. Comparison of the regulatory function of toll-like receptor 4 (TLR4)^{-/-} B cell with that of wild-type (WT) B cells after ultraviolet (UV) B irradiation. Purified WT T cells with WT B cells or TLR4^{-/-} B cells were adoptively transferred into Rag-1^{-/-} recipient mice before oxazolone (OXA) sensitization and UVB irradiation. Ear swelling (**a**, **b**) and T cell infiltration (**c**) were assessed 48 h after OXA challenge. The data shown are the mean $\pm$ standard error of the mean from one experiment with two repetitions yielding similar results. * $p < 0.05$.

immunosuppression, the mechanism underlying this is not well defined. In this study, we showed that regulatory B cells contributed to UVB-induced immunosuppression in CHS, and were important for inhibiting antigen-specific T cell proliferation following OXA challenge. Moreover, we found that the TLR4 pathway was required for regulatory B cell expansion with respect to UVB irradiation.

UVB has been reported to inhibit CHS by modulating immunosuppression (28, 29). A large number of immunosuppressive events are initiated after UVB irradiation. At the cellular level, UVB-induced immunosuppressive cells are essential for maintaining systemic immunosuppression (1, 30). Studies performed by Yanaba et al. showed that regulatory B cells restricted the CHS response through IL-10 production (13). In our study, Rag1^{-/-} mice that received T cells alone not only showed a significantly increased CHS response as compared with matched control mice, but also showed lower sensitivity to UVB phototherapy. As regulatory B cells were found to be

expanded after UVB irradiation, and B cells isolated from UVB-irradiated mice showed an enhanced inhibitory effect on T cells as compared with B cells isolated from non-irradiated mice, we concluded that regulatory B cells, which were induced by UVB irradiation, contributed to UVB-induced systemic immunosuppression in CHS.

UVB-induced regulatory T cells have been widely considered to mediate UVB-induced immunosuppression by inhibiting T effector cells (31–33). However, because of the lack of regulatory T cell depletion experiments in our study, we cannot exclude a suppressive role of regulatory T cells in our research model. Sun et al. reported that regulatory B cells were critical in the bone union process by stimulating regulatory T cells (34). Song et al. suggested that regulatory B cells were mainly responsible for the amplification of regulatory T cells by sirolimus (35). Liu et al. observed that, in chronic hepatitis B virus infection, regulatory B cells could enhance regulatory T cell function and influence

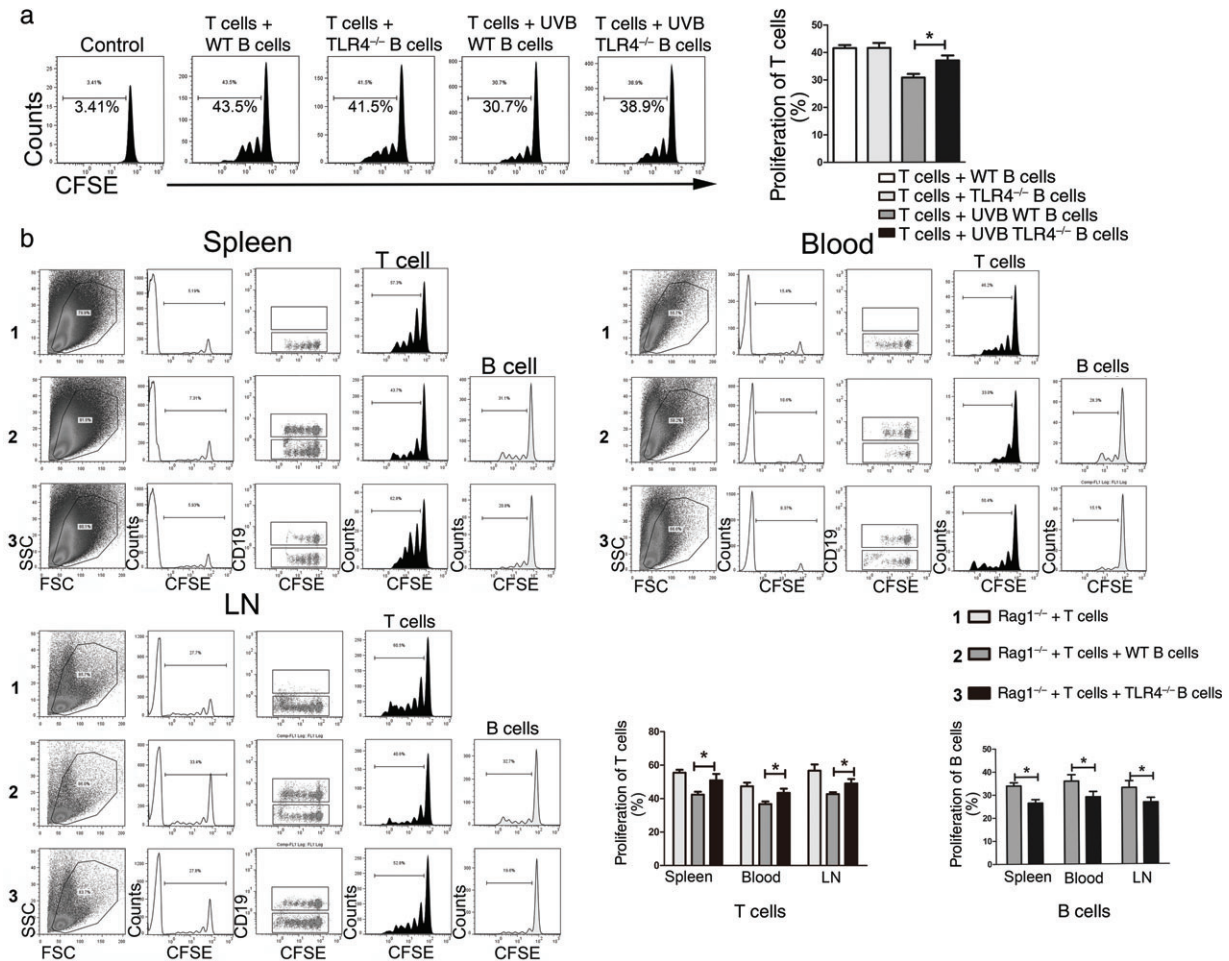


Fig. 7. The inhibitory effects of toll-like receptor 4 (TLR4)^{-/-} B cells and wild-type (WT) B cells on T cells after ultraviolet (UV) B irradiation. **(a)** B cells from WT mice and TLR4^{-/-} mice that, with or without UVB irradiation, were isolated and co-cultured with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled T cells in the presence of anti-CD3 and anti-CD28 stimulation. T cell proliferation was assessed by flow cytometry. **(b)** Rag1^{-/-} mice received CFSE-labelled T cells, CFSE-labelled T cells and WT B cells, or CFSE-labelled T cells and TLR4^{-/-} B cells. These mice were challenged with oxazolone and exposed to UVB radiation. The proliferation of T cells and B cells was assessed by flow cytometric assay. Data are from one experiment with 5 mice and two repetitions yielding similar results. **p* < 0.05. LN, lymph node.

immune tolerance (36). Whether regulatory T cells and regulatory B cells underwent synergistic interactions in maintaining UVB-induced immunosuppression was still undetermined in our study. Further studies would be needed to clarify the interplay between regulatory B cells and regulatory T cells in UVB-induced systemic immunosuppression.

Our results not only confirmed the suppressive function of regulatory B cells in CHS, but also indicated that UVB is a potent inducer of regulatory B cells. In our study, the expression of TLR4 on B cells was upregulated after UVB irradiation. TLR4^{-/-} mice showed significantly decreased regulatory B cells as compared with WT mice in response to UVB irradiation. Moreover, TLR4 deficiency in B cells

not only impaired the inhibitory function of B cells from UVB-irradiated mice, but also diminished the therapeutic effect of UVB on CHS. From our findings, we conclude that the UVB–TLR4 axis plays an important role in the generation of regulatory B cells and amelioration of CHS.

TLRs, which recognize pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns, play a key role in recognizing microorganisms, initiating innate immunity, and promoting adaptive immune responses (37, 38). TLR4 is important for recognizing PAMPs, especially LPS present in gram-negative bacteria. Recent studies also indicated that some damage-associated molecular patterns released from

injured cells after UVB irradiation were also candidates for triggering TLR4 signalling (39–41). Moreover, the *Lps^d* allele, which is homologous to *TLR4*, has been found to be required for UVB-induced immunosuppression (42, 43). Furthermore, Lewis et al. reported that TLR4 mediated UVB-induced suppression primarily by the generation of regulatory T cells (25). Such evidence suggests that TLR4 ligands released after UVB irradiation play an important role in mediating UVB-induced immunosuppression.

Interestingly, in addition to B cell receptor or CD40 ligation, TLR signalling plays an important role in B cell differentiation and activation. Liu et al. showed that the activation of STAT3 and extracellular signal-regulated kinase was important for IL-10 production by human B cells following TLR stimulation (44). Yanaba et al. reported that IL-10 secretion by B cells was induced by TLR4 signalling through MyD88-dependent pathways (23). Data from Lampropoulou et al. indicated that B cells activated via TLR4 suppressed T cell proliferation in autoimmune disease (45). Results from previous studies showed that TLR pathways, especially the TLR4 pathway, played a vital role in the generation of regulatory B cells. In our study, we confirmed a role of TLR4 signalling in the induction of regulatory B cells, and indicated that TLR4-induced regulatory B cells contributed to UVB-mediated immunosuppression in CHS. Although *TLR4^{-/-}* mice generated significantly fewer regulatory B cells than WT mice in response to UVB irradiation, the numbers of regulatory B cells were increased in UVB-irradiated *TLR4^{-/-}* mice as compared with non-irradiated *TLR4^{-/-}* mice (Fig. 5). In addition, the therapeutic effect of UVB on CHS in mice with *TLR4^{-/-}* B cells was more pronounced than that in mice without B cells (Fig. 6). These data suggest that TLR4 signalling was important but was not the only pathway for generation of regulatory B cells after UVB irradiation. Bernard et al. reported that TLR3 was also necessary for UVB-induced immunosuppression (46). There is also considerable evidence that prostaglandin, PAF (platelet activating factor), *cis*-urocanic acid, IL-10 and IL-4 are necessary for activating UVB-induced systemic immunosuppression (47, 48). Although the interplay between these UVB-induced mediators and regulatory B cells is not completely understood, we cannot exclude the possibility that these compounds are candidates for inducing regulatory B cells after UVB irradiation. Moreover, a number of other observations in the literature suggest that various candidates for activating TLR4, including oxidized lipids, heat-shock proteins, the S100 family proteins, high-mobility group box 1, and hyaluronic acid, are released into serum after UVB irradiation (39). In

our study, we did not identify which agonist was most important for generating regulatory B cells in our model. Further studies are also needed to determine which TLR4 ligand is critical for TLR4-dependent UVB-mediated immunosuppression.

In summary, our study shows that TLR4-mediated generation of regulatory B cell is involved in the immunosuppressive effect of UVB on CHS. In addition to the previous findings that regulatory T cells are required for UVB-induced systemic immunosuppression in contact dermatitis, our study provides evidence that activation of regulatory B cells is also important for the systemic immunosuppression induced by UVB. Moreover, the TLR4 pathway in B cells activated after UVB radiation probably plays an important role in the generation of regulatory B cells. Our results yield insights into the mechanisms by which UVB radiation mediates systemic immunosuppression. Efforts to activate the TLR4 pathway in B cells will enhance the generation of regulatory B cells, and may prove fruitful for enhancing photoimmunosuppression by UVB.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The distribution of IL-10⁺B cells in peripheral B cell subsets. B cell was gated from CD19⁺ cell subset, transitional T1 B cell was gated from (CD93⁺ CD23⁻ IgM^{high}) cell subset, transitional T2 B cell was gated from (CD93⁺ CD23⁺ IgM^{high}) cell subset, transitional T3 B cell was gated from (CD93⁺ CD23⁻ IgM^{low}) cell subset, Follicular B cell (FO) was gated from (CD93⁻ CD21^{int} IgM⁺) cell subset, MZ B cell was gated from (CD93⁻ CD21^{high} CD23⁻) cell subset, MZP B cell was gated from (CD93⁻ CD21^{high} CD23⁺) cell subset.

Figure S2. Assessment the function of IL-10⁺B cells. **(a)** IL-10⁺B cells or IL-10⁻B cells were isolated from the spleen of UVB irradiated WT mice. Purity of the isolated B cell was assessed by flow cytometry assay. **(b)** IL-10⁺B cells and IL-10⁻B cells were isolated and cultured with T cells (T: B = 1:1, 4 × 10⁵/well) in the presence of anti-CD3 Ab (1 µg/ml) and anti-CD28 Ab (0.5 µg/ml) in a 96-well

plate for 72 h. Proliferation of CD4⁺T cells were assessed by flow cytometry assay. (***) $p < 0.001$). Data are pooled

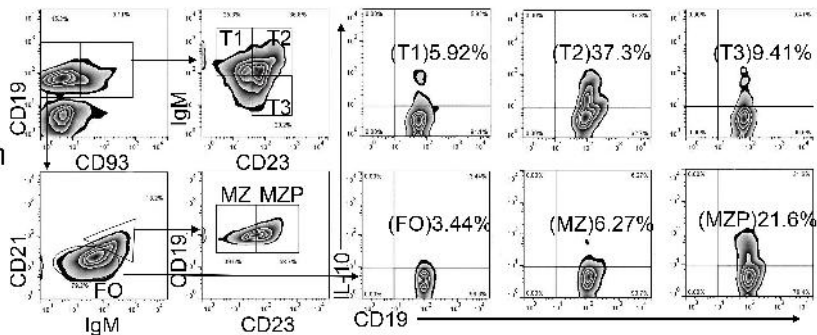
from three independent experiments and expressed as mean $\pm$ SEM.

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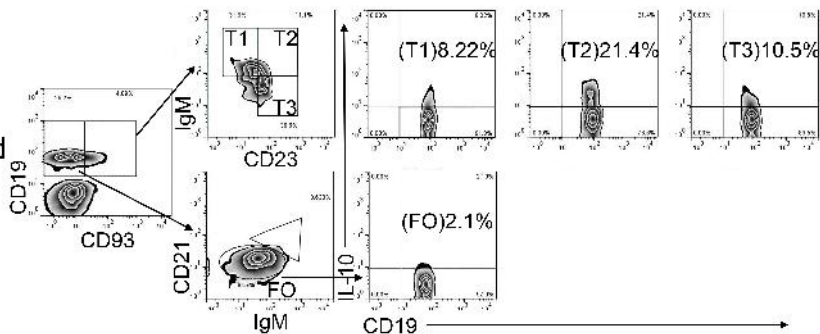
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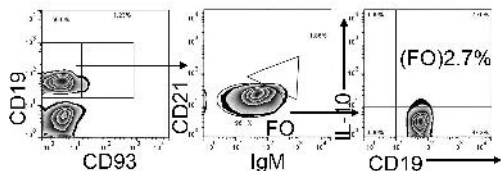
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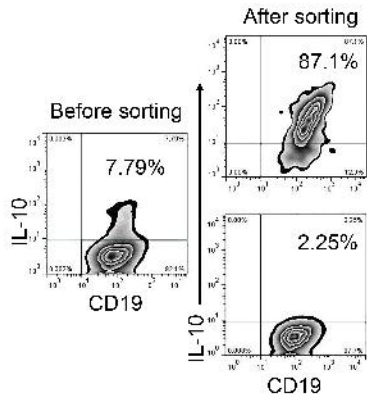
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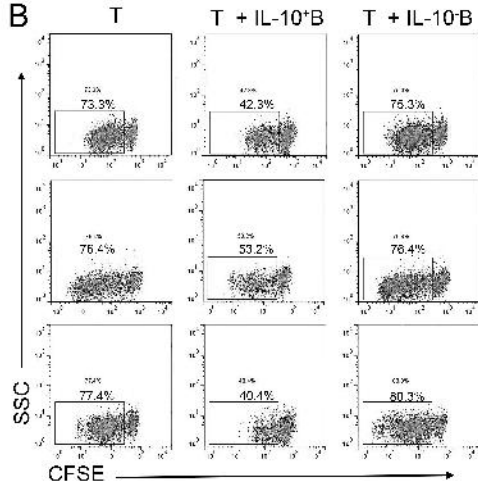
LN



A



B



T Cell
 T Cell + IL-10⁺B Cell
 T Cell + IL-10⁻B Cell

