Photochemical & Photobiological Sciences

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Journal:	*Photochemical & Photobiological Sciences*
Manuscript ID:	PP-ART-10-2014-000399.R1
Article Type:	Paper
Date Submitted by the Author:	13-Jan-2015

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The alternative complement component factor B regulates UV-induced oedema, systemic suppression of contact and delayed hypersensitivity, and mast cell infiltration into the skin

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Abstract

Ultraviolet (UV) wavelengths in sunlight are the prime cause of skin cancer in humans with both the UVA and UVB wavebands making a contribution to photocarcinogenesis. UV has many different biological effects on the skin that contribute to carcinogenesis, including suppression of adaptive immunity, sunburn and altering the migration of mast cells into and away from irradiated skin. Many molecular mechanisms have been identified as contributing to skin responses to UV. Recently, using gene set enrichment analysis of microarray data, we identified the alternative complement pathway with a central role for factor B (fB) in UVA-induced immunosuppression. In the current study we used mice genetically deficient in fB (fB-/- mice) to study the functional role of the alternative complement pathway in skin responses to UV. We found that fB is required for not only UVA but also UVB-induced immunosuppression and solar-simulated UV induction of the oedemal component of sunburn. Factor B-/- mice had a larger number of resident skin mast cells than control mice, but unlike the controls did not respond to UV by increasing mast cell infiltration into the skin. This study provides evidence for a function role for fB in skin responses to UV radiation. Factor B regulates UVA and UVB induced immunosuppression, UV induced oedema and mast cell infiltration into the skin. The alternative complement pathway is therefore an important regulator of skin responses to UV.

Introduction

Ultraviolet (UV) radiation that reaches the surface of Earth from the sun is the main cause of skin cancer in humans. UV radiation has diverse damaging effects on the skin that contribute to skin carcinogenesis, including immunosuppression, genetic damage and dysregulation of the cell cycle. UV radiation also causes sunburn, which can be observed as an acute thickening and reddening of the skin [1] [2]. UV-induced immunosuppression is both damaging as it contributes to skin carcinogenesis, and beneficial as it may provide some protection from colitis and T cell mediated autoimmune diseases such as multiple sclerosis [3] [4]. Both the UVA (320 – 400 nm) and UVB (290 – 320 nm) wavebands are likely to contribute to photocarcinogenesis [5] [6]. They both suppress immunity, and as the action spectra for these wavebands do not overlap, the mechanisms for UVA and UVB-induced immunosuppression are likely to differ [6]. While UV can cause both local and systemic immunosuppression, the mechanisms involved are likely to differ [3]. Local is where the antigen is applied locally to the UV irradiated site while systemic is where the antigen is applied to an unirradiated skin site distal to the exposed skin. UV immunosuppression is frequently studied by contact or delayed-type hypersensitivity (CHS and DTH respectively). Contact is when a chemically reactive hapten is applied topically to the surface of the skin whereas DTH involves injection of a protein antigen into the dermis. During the induction of CHS, antigen presenting cells in the epidermis first encounter antigen. In contrast, initiation of DTH involves antigen presenting cells from the dermis. The mechanisms underlying suppression of CHS and DTH are therefore likely to be different. Indeed, it has been shown that different soluble factors are involved in UV suppression of CHS and DTH [7] and that the UV dose responses differ [8]. Moreover,

UV suppression of cytotoxic T cell activation appears to involve yet a different mechanism to suppression of DTH [9].

The mechanisms by which UVA and UVB suppress immunity are complex, commencing with absorption of the UV photons in the skin. Many mediators have been described to be involved in this process and are dependent on the type of immune response and wavelengths being studied [10 11]. Previously we used gene set enrichment analysis of microarray data to identify pathways that may be different in UVA compared to UVB-induced suppression of systemic CHS [12]. Our results indicated that the alternative complement pathway is a sensor of UVA but not UVB-induced damage that leads to immunosuppression. Real-time RT-PCR identified the genes for complement component 3, properdin, and complement factor B (fB) as being particularly important for mediating UVA-induced systemic immunosuppression. In the current study we used fB knockout (fB-/-) mice to examine the function of the alternative complement pathway in UVA and UVB-induced systemic suppression of CHS and DTH. Here we confirm that fB is involved in UVA-induced systemic immunosuppression, but unexpectedly also found that it is involved in UVB induced immunosuppression as well as UV-induced oedema.

Materials and Methods

Mice

Factor B knockout (fB-/-) mice produced by targeted deletion[13] and back-crossed for at least nine generations onto a C56BL/6 background[14] were provided by Dr Joshua Thurman (University of Colorado). They were breed at the University of Sydney. fB-/- and C57BL/6 mice (Animal Resource Centre, Perth, WA, Australia) aged 8-10 weeks at the start of experiments were used with approval from the University of Sydney animal ethics committee (approval number K14/2-2010/3/5233). Mice were supplied food and water *ad libitum*.

UV irradiation

Mice were removed of dorsum fur 24 h before irradiation with animal clippers (Oster, TN) and an electric razor (Remington, Austria). During irradiation, mice were restrained within black Perspex boxes with a quartz lid, and ears and heads were protected from the UV with black Perspex.

A 1000 W xenon arc lamp solar simulator (Oriel, Stanford, CT) was used to produce UVA, UVB or solar-simulated UV (ssUV) spectra. For the broadband UVA spectra, the source was filtered with two 200-400 nm dichroic mirrors and a UVB blocking filter (CVL Laser, Albuquerque, NM). This UVA spectrum had a wavelength range of 325 to 420nm and peak irradiance at 370nm. UVB (below 320nm) and UVC (below 290nm) contaminated the spectra by less than 0.01% and 0.001%, respectively. The spectral output has been published previously [15]. The UVB spectrum was created with the same dichroic mirrors, but using a 310 nm narrowband

interference filter (CVL Laser, Albuquerque, NM). The UVB spectra had a peak irradiance at 312nm, and a halfband width of ~15 nm. UVA (above 320nm) and UVC (below 290nm) contaminated the spectra by ~23% and 0.61%, respectively. This spectrum has been published previously [15]. The ssUV spectrum obtained using an atmospheric attenuation filter (Oriel) had a peak irradiance at 380nm and consisted of 8.1% UVB (290-320nm) and 91.9% UVA (320-400 nm) contaminated by less than 0.01% UVC (below 290nm).

Spectral output and intensity was measured with a calibrated OL-754 spectroradiometer (Optronics Laboratories, Orlando, FL). An IL700 broadband radiometer (International Light Technologies, Newburyport, MA) calibrated against the source with the spectroradiometer was used routinely to monitor fluctuations in output.

Contact and Delayed Hypersensitivity and Oedema

Three days after the shaved dorsums were exposed to a single or final dose of UV, mice were immunised. For contact hypersensitivity (CHS) the shaved, unirradiated abdominal skin was immunised with 30µl of the chemical hapten 2,4-dinitro-1-fluorobenzene (DNFB; Sigma-Aldrich; 0.5% v/v in 4:1 acetone:olive oil). Five days later, the ears of the mice were challenged with 15µl per ear of DNFB (0.25% v/v in the same diluent), applied evenly to the dorsal and ventral surfaces. For delayed type hypersensitivity (DTH), mice were injected on their abdomens subcutaneously with 200µg ovalbumin (ova; Sigma-Aldrich, St Louis, USA) and 40µg saponin (Sigma-Aldrich) in saline as we have described previously [9]. Mice were assessed for a DTH reaction 7 days after immunisation by intradermally injecting 50µg ova in 10µl saline into each ear. For both CHS and DTH, ears were measured

before ear challenge and at 24 hr after challenge using micrometer calipers (Interapid, Switzerland). Any increase due to non-specific ear inflammation was assessed in naïve, unimmunised mice. The CHS or DTH reactions were determined by the difference between pre and post ear thickness measurements after accounting for non-specific inflammation.

Double skinfold thickness of back skin was measured before and at various times after exposure to ssUV with a spring-loaded engineer's micrometer (Mercer, St. Albans, Herts., England). The increase in double skinfold thickness in response to ssUV was the oedemal component of sunburn. This is often used to assess sunburn in mice as the dose response for oedema in mice closely matches that of sunburn in humans, and erythema is difficult to assess in mice [16, 17].

Mast Cell Determination

Groups of wildtype and fB-/- mice were exposed (or not) to 8Jcm^{-2} ssUV. Six hours later the skin was isolated and 7μm sections of paraffin-embedded formalin fixed skin was stained with toluidine blue to identify and quantitate mast cells. This dose and timing is sufficiently high enough to result in the rapid recruitment of mast cells into UV-exposed skin [18]. The numbers of dermal mast cells were then counted and the length of skin determined using ImageJ software (http://rsbweb.nih.gov/ij/).

Data Analysis

CHS, DTH and mast cell data are shown as median with interquartile range. Kruskal-Wallis ANOVA with Dunn's Multiple Comparison Test was used for statistical comparison between multiple groups. The UV-induced oedema time course data was analysed using repeated measures ANOVA with Tukey's test for multiple

comparisons and is shown as mean and SEM. Data was analysed using Prism software (version 6, GraphPad Software, La Jolla, CA), with $P<0.05$ considered statistically significant.

Results

Mice deficient in Factor B are resistant to UV suppression of Contact and Delayed Type Hypersensitivity

Contact hypersensitivity occurs when a hapten (DNFB) is applied topically to the skin where it binds to self-carrier proteins to initiate immunity in the epidermis. In these experiments the antigen was applied to abdominal skin distal to the irradiated dorsal trunk site, therefore assessing systemic immunity (Fig. 1). The wildtype control mice were systemically immunosuppressed by both UVA and UVB. In contrast, the fB-/- mice were not immunosuppressed by either waveband.

Delayed type hypersensitivity is a response to an intradermally injected protein antigen (ova) so that the immunity is initiated in the dermis. As for the CHS assay, systemic immunity was assessed by injecting the antigen into abdominal skin distal to the irradiated site (Fig. 2). While the DTH response was suppressed in wildtype mice irradiated with UVA or UVB, neither waveband suppressed immunity in the fB-/- mice. Therefore fB appears to be required for both UVA and UVB-induced systemic suppression of CHS and DTH.

Mice deficient in Factor B are relatively less susceptible to UV-induced oedema

Due to their pink skin colour, oedema rather than erythema is frequently used to assess UV induced sunburn in mice. Wildtype control mice exposed to $6Jcm^{-2}$ ssUV developed a large oedematous response at the irradiated skin site that peaked on day 4 and returned nearly to baseline by day 7 (Fig. 3). The response in the fB-/- mice was significantly reduced about 10 fold compared to the controls. Increasing the ssUV dose to $8Jcm^{-2}$ did not have a statistically significant effect on this response in wildtype mice. However it increased oedema in the fB-/- mice to 53% of the control

group at the peak on day 4 so that there was no longer any significant difference between the genotypes.

UV irradiation fails to increase dermal mast cells in mice deficient in Factor B

Mast cells that infiltrate UV-irradiated skin contribute to both UV-induced immunosuppression and oedema. Hence we examined these cells in UV irradiated skin. As expected, ssUV exposure of wildtype mice caused significant mast cell infiltration into the exposed skin. In contrast UV did not significantly influence mast cell numbers in the skin of fB-/- mice (Fig. 4). However fB-/- mice had a significantly larger number of resident mast cells than the control mice. Thus Factor B appears to regulate the number of resident and UV-induced inflammatory mast cells in the dermis.

Discussion

Immune surveillance is in part initiated and mediated by the complement system which recognizes altered cells and produces signals that contribute to the immune response against the altered cell [19]. There are 3 interconnecting pathways in the complement system. IgM or IgG antibodies binding to their specific antigen activates the first component of the Classical Complement pathway, C1. This causes a cascade of molecular events resulting in an intermediate step being cleavage of C3 into its active fragments C3a and C3b with further downstream events then occurring. In the lectin pathway carbohydrate pattern recognition molecules cause the same C3 to be cleaved via a different series of molecular events. The third pathway is the alternative pathway in which C3 undergoes "tick-over" where there is a low level hydrolysis exposing binding sites for factor B (fB), a critical mediator in the activation of this complement cascade. Thus C3 is central to the complement system and can be activated via a number of different pathways (reviewed by [19]). As multiple immune events can be regulated by the complement system, it was perhaps not surprising that we identified the alternative complement pathway as being critical for UVA-induced immunosuppression by analysis of mRNA pathways activated by UVA [12]. UVB-induced activation of C3 has also been shown to be important for local immunosuppression [20 21].

In this study we demonstrated a functional role for fB in both UVA and UVB induced immunosuppression, ssUV-induced oedema, and mast cell infiltration into ssUV irradiated skin. Our previous microarray experiments identified the alternative

complement pathway as being involved in UVA immunosuppression but did not detect its role in UVB immunosuppression. The reasons for this are not clear. However we only examined changes in mRNA expression at a single time point and therefore it is possible that UVB effects on alternative complement components may have occurred at a different time. UVA immunosuppression has a different dose and time response to UVB immunosuppression. Whereas UVA has a Gaussian or bell-shaped dose responsive effect on immunosuppression, UVB has a linear dose response, at least up to the physiological doses that can be examined without causing extensive sunburn [22]. Additionally the time course of UVA and UVB immunosuppression differ [23]. In humans, UVB induced immunosuppression can be observed within 24h of exposure while UVA immunosuppression requires 48h [24] suggesting that the molecular mechanisms responsible for immunosuppression by these wavebands are different and/or require different amounts of time to become activated. Indeed in the DTH experiments we used different time courses to demonstrate UVA and UVB immunosuppression as we find that different times are required to detect suppression by these different wavebands. Hence it is likely that the limited time course in our microarray experiments may have been responsible for identification of a role for the alternative complement pathway in UVA but not UVB immunosuppression.

We used different doses of UV for the different biological end points we studied. We have previously found that a single exposure to 1.8 Jcm^{-2} UVA is optimal for this waveband to suppress immunity [25] while the lower dose of 0.15 Jcm^{-2} UVB is sufficient to suppress immunity in the absence of sunburn. To induce the oedemal component of sunlight higher doses are required and we found that while fB regulates

oedema induced by 6 Jcm^{-2} ssUV, a deficiency in fB was not able to significantly inhibit oedema in response to the higher dose of 8 Jcm^{-2} ssUV. The reason for this is unclear, fB may have a limited ability to regulate UV-induced oedema or a different mechanism may dominate at the higher dose. The role of fB in sunburn formation is not clear, however C3 is deposited on sunburn cells, suggesting activation of the alternative complement pathway [26]. The alternative complement pathway is one of the molecular mechanisms that can cause inflammation. Inflammation is part of the oedemal response to UV. It is possible that fB regulation of inflammation may influence UV-induced oedema.

Dermal mast cells are a major cellular mediator of UV-induced suppression of adaptive immunity [27 28]. One way mast cells mediate immune suppression is by migrating in response to UV-established chemokine gradients into and away from the irradiated skin [18]. This alteration to mast cell traffic is important not only for photocarcinogenesis [29] but is also associated with successful phototherapy in polymorphic light eruption patients [30]. Our results suggest that activation of the alternative complement pathway is another potential mechanism by which mast cell movements are regulated by UV. While exposure to ssUV caused a significant increase in dermal mast cells 6h later in wild type mice as expected, this failed to occur in fB-/- mice. Exactly how activated products of the complement cascade could be involved in mast cell recruitment to skin remains unclear. One possibility is that mast cells migrate towards activated complement proteins C3a and C5a for which they express the receptors [31]. Another interesting finding to come out of our studies is that Factor B deficient mice had significantly higher baseline densities of dermal mast cells. This may explain why these mice were resistant to UV-induced oedema because

mast cells are required for protection from cutaneous UV-induced inflammation [32]. Precisely how an absence of Factor B leads to an increase in resident dermal mast cells remains to be determined.

In conclusion, many different molecular pathways are dysregulated by UV and contribute to skin responses [11]. This study indicates that fB and the alternative complement pathway contribute to UV-induced immunosuppression, oedema and mast cell infiltration into the skin.

Acknowledgements

We acknowledge financial assistance for our research from The Cure Cancer Australia Foundation, The National Health and Medical Research Council of Australia, The New South Wales Cancer Council and Sydney Medical School Foundation.

Figure Legends

Fig. 1. CHS responses are not suppressed by UVA or UVB in fB-/- mice. Groups of wildtype (WT) or fB-/- mice were given a single exposure to 1.8 Jcm^{-2} UVA or 0.15 Jcm^{-2} UVB on their shaved dorsal trunk and were contact sensitized 3 days later on their abdomen. CHS data shown from a pool of 5 experiments that were each normalised to the unirradiated (NoUV) wildtype control to correct for interexperimental variations in magnitude of the response. Each experiment used mice of a single sex (3 female, 2 male) so that the final pooled data is a mixture of 13-17 male and female mice. Median and interquartile range shown. Statistical analysis by Kruskal-Wallis ANOVA with Dunn's Multiple Comparison Test compared to the unirradiated control for each genotype. n.s. = not significant.

Fig. 2. DTH responses are not suppressed by UVA or UVB in fB-/- mice. Groups of wildtype (WT) or fB-/- mice were given a single exposure to 1.8 Jcm^{-2} UVA or 3 exposures on consecutive days to 0.15 Jcm^{-2} UVB on their shaved dorsal trunk. Mice were immunised on their abdomen with ova 3 days after the single UVA or final UVB exposures. DTH data shown as a pool of 2 experiments that were each normalised to the unirradiated (NoUV) wildtype control to correct for interexperimental variations in the magnitude of the response. All mice used were females, with 4-9 mice per group. Median and interquartile range shown. Statistical analysis by Kruskal-Wallis ANOVA with Dunn's Multiple Comparison Test compared to the unirradiated control for each genotype. n.s. = not significant.

Fig. 3. Reduced UV-induced oedema in fB-/- mice. Groups of 5-8 male wildtype (WT) and fB-/- mice were exposed to a single dose of solar-simulated UV (ssUV) on their shaved dorsal trunk on day 0. The increase in double skinfold thickness compared to the pre-irradiation value was measured for 7 consecutive days following exposure. The mean and SEM are shown. Where the SEM is not obvious it is too small to be seen. Difference between WT and fB-/- mice exposed to 6Jcm^{-2} (closed circles and closed squares respectively) statistically significant over time course ($P<0.01$, repeated measures ANOVA with Tukey's multiple comparison test). No significant difference between WT and fB-/- mice exposed to 8Jcm^{-2} (open circles and open squares respectively).

Fig. 4. UV does not induce mast cell infiltration into the skin of fB-/- mice. Groups of 6-9 male and female wildtype (WT) and fB-/- mice matched for sex and age were exposed to a single dose of 8 Jcm^{-2} solar-simulated UV (ssUV) on their shaved dorsal trunk. The irradiated skin was removed 6 h after exposure. Toluidine blue positive mast cells were enumerated in the dermis per mm skin. The results show a pool of 2 experiments that were each normalised to the unirradiated (NoUV) wildtype control to correct for interexperimental variations. Median and interquartile range shown. Statistical analysis by Kruskal-Wallis ANOVA with Dunn's Multiple Comparison Test, n.s. = not significant. Comparisons on figure compared to unirradiated control for each genotype. Significant difference ($P<0.001$) between the unirradiated genotypes.

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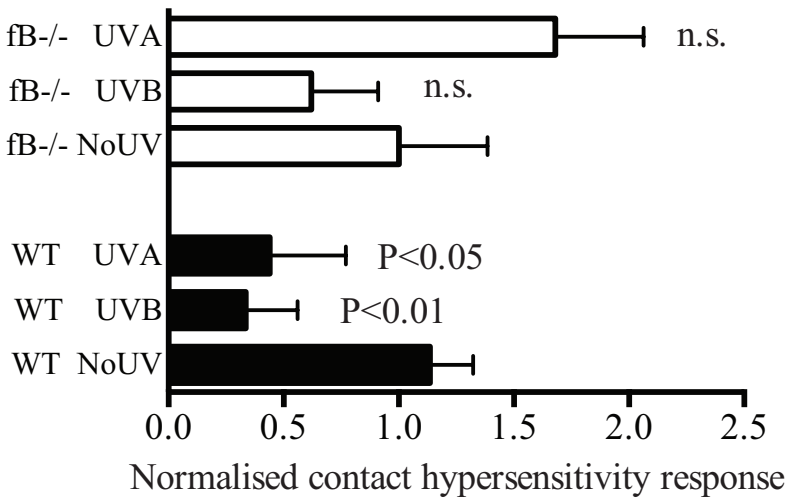
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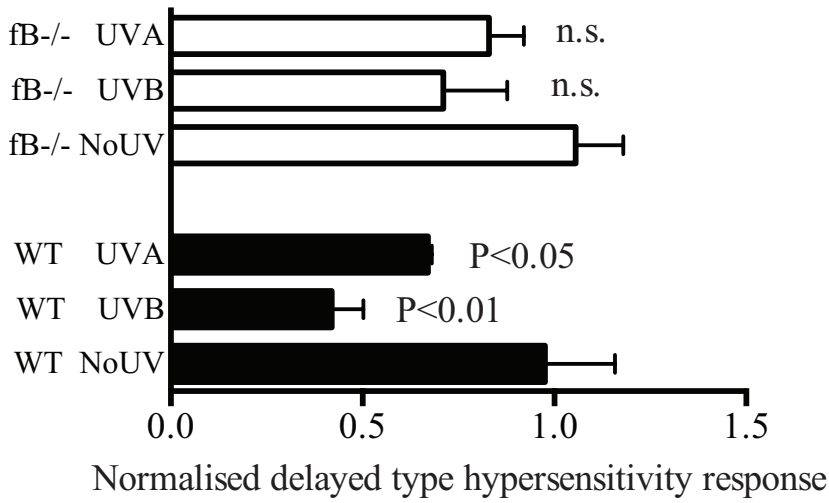
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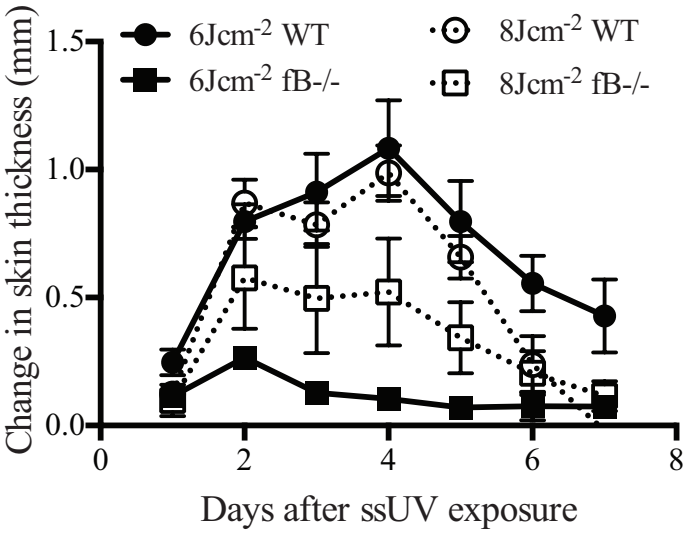
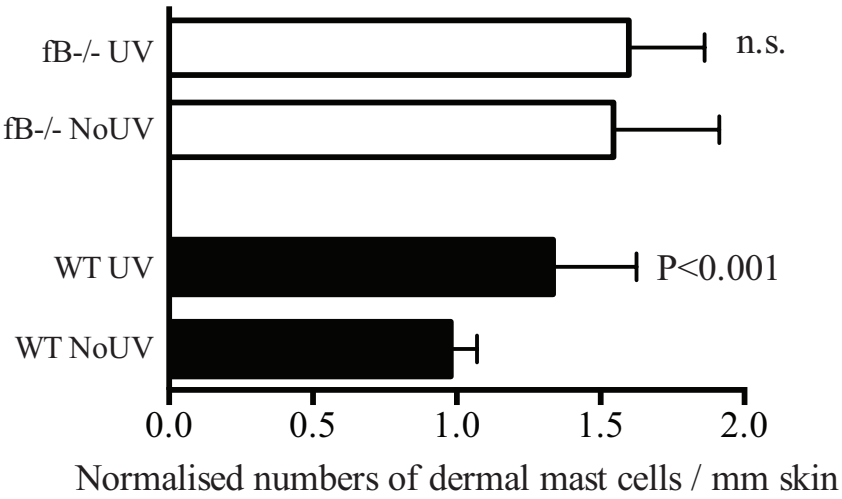
1.5
Normalised delayed type hypersensitivity response


6Jcm-2 WT
6Jcm-2 fB-/-
8Jcm-2 WT
8Jcm-2 fB-/-
Change in skin thickness (mm)
1.5
1.0
0.5
0.0
0
2
4
6
8
Days after ssUV exposure


fB-/- UV
fB-/- NoUV
WT UV
WT NoUV
n.s.
P<0.001
0.0
0.5
1.0
1.5
2.0
Normalised numbers of dermal mast cells / mm skin