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# Daily very low UV dose exposure enhances adaptive immunity, compared with a single high-dose exposure. Consequences for the control of a skin infection

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## Summary

**Ultraviolet radiation (UVr) promotes several well-known molecular changes, which may ultimately impact on health. Some of these effects are detrimental, like inflammation, carcinogenesis and immunosuppression. On the other hand, UVr also promotes vitamin D synthesis and other beneficial effects. We recently demonstrated that exposure to very low doses of UVr on four consecutive days [repetitive low UVd (rlUVd)] does not promote an inflammatory state, nor the recruitment of neutrophils or lymphocytes, as the exposure to a single high UV dose (shUVd) does. Moreover, rlUVd reinforce the epithelium by increasing antimicrobial peptides transcription and epidermal thickness. The aim of this study was to evaluate the adaptive immune response after shUVd and rlUVd, determining T-cell and B-cell responses. Finally, we challenged animals exposed to both irradiation procedures with *Staphylococcus aureus* to study the overall effects of both innate and adaptive immunity during a cutaneous infection. We observed, as expected, a marked suppression of T-cell and B-cell responses after exposure to an shUVd but a novel and significant increase in both specific responses after exposure to rlUVd. However, the control of the cutaneous *S. aureus* infection was defective in this last group, suggesting that responses against pathogens cannot be ruled out from isolated stimuli.**

**Keywords:** bacterial infection; immune stimulation; immune suppression; skin; ultraviolet radiation.

## Introduction

Ultraviolet radiation (UVr), contained within sunlight that reaches the Earth's surface, promotes several changes and alterations both on exposed skin and internal organs, especially on immune organs. The molecular events triggered by UVr on exposed cells are well known, they include DNA damage (formation of cyclobutane pyrimidine dimers and 6,4-pyrimidine-pyrimidone or 6,4-photoproducts), *trans*-urocanic acid isomerization, reactive oxygen and nitrogen species production and mitogen-activated protein kinase cascade activation, among others.[1–4] These molecular events lead to cellular and tissue alterations, such as local inflammation, systemic immunosuppression and skin cancer development, including both melanoma and non-melanoma skin cancer.[5–8] The extent of the damage depends on some characteristics of the exposed individual, such as the skin


 

pigmentation, and on the dose of UVr that effectively reaches the skin.

Diverse animal models have been extensively employed to study UV-induced alterations on health. Almost a century ago, Findlay described the ability of UV light to induce skin cancer in albino mice chronically exposed to a quartz mercury-vapour lamp.[9] Plenty of experimental conditions were analysed since then in animal models; we would like to highlight the following concepts: (i) chronic exposure to UV light leads to non-melanoma skin cancer development; this observation is valid when animals were exposed to UVr doses above the minimal erythemal dose (MED, a dose that promotes visible damage by itself) but also for sub-erythemal doses (around half the MED).[10,11] (ii) Acute exposure to UVr promotes a deficient adaptive immune response; this well-known immunosuppressive effect has been demonstrated by exposing animals to a single UV exposure (above MED) as well as to several exposures to low UV doses (around half the MED and applied on a few consecutive days). Cutaneous T-cell response to different sensitizers (e.g. oxazolone, 1-fluoro-2,4-dinitrobenzene) is the most common technique employed to report the UV-induced immunosuppression.[12,13] (iii) Exposure to UV light has beneficial effects on health; besides the fact that human exposure to sunlight is responsible for vitamin D formation, animal exposure to very low doses of UVr (a tenth of the MED) induces the production of antimicrobial peptides on the skin, probably through the synthesis of that vitamin.[14] (iv) It has been stated that exposure to UVr may affect immune responses to pathogens and vaccines;[15] however, the exact role of the different doses mentioned above on skin infections has been poorly studied.

We have recently published a work comparing the effects of different UV exposures on skin innate immunity, using two animal models.[16] One comprised in exposing the animals to a single high UV dose (shUVd, 2 MED), simulating a harmful exposure to the sun. The second one consists of repetitive low UV doses (rlUVd, 0.1 MED), representing short daily exposures. We observed, as described previously, that exposure to an shUVd promoted local inflammation (by the production of pro-inflammatory cytokines and chemokines both in epidermis and dermis and recruiting neutrophils and lymphocytes), epidermal damage and a slight increase in antimicrobial peptides, without affecting vascularization of the skin. On the other hand, exposure to rlUVd was unable to induce any sign of local inflammation, although it promoted a marked increase in antimicrobial peptide transcription and in vascularization (by increasing the transcription of vascular endothelial growth factor in epidermis and dermis) and promoted a slight epidermal hyperplasia. We have proposed that rlUVd promotes reinforcement of the barrier function of the skin, without generating deleterious effects on the tissue.

Considering the opposing effects of shUVd and rlUVd on skin innate immunity, we decided to study the effects of both types of exposure on systemic adaptive immunity and on the response to a pathogen. First, we evaluated T-cell populations from skin-draining lymph nodes and spleen of animals exposed to shUVd and rlUVd using *in vitro* assays. Then, specific *in vivo* T-cell and B-cell responses were analysed through contact hypersensitivity (CHS) reaction to oxazolone and antibody production against tetanus toxoid, respectively. Finally, we studied both innate and adaptive immune responses during an experimental *Staphylococcus aureus* cutaneous infection,[17] established after shUVd or rlUVd exposure.

## Materials and methods

### *Mice and irradiation schemes*

Male Crl:SKH1-hrBR hairless mice between 7 and 9 weeks of age (20–25 g), purchased from Charles River Laboratories (Wilmington, MA), were housed in a 12 hr light/12 hr dark cycle and maintained with water and food *ad libitum*. The animals were irradiated with UV light using an 8W UVM-28 mid-range (302 nm) lamp from Ultraviolet Products (UVP, Upland, CA), which emits most of its energy within the UVB range (emission spectrum range 280–370 nm), with a peak at 302 nm, including a 20–30% amount of UVA. The lamp irradiance was measured to be 1·2 mW/cm$^2$ using a UVX radiometer (UVP).

The mice were irradiated on their backs with a single high dose of UVr (400 mJ/cm$^2$, corresponding to 2 MED) simulating a harmful exposure, or with four repetitive low doses of UVr (20 mJ/cm$^2$, corresponding to 0·1 MED), over 4 consecutive days, simulating daily exposures. The animals were exposed to the lamp for 6 min and 4 seconds to achieve the 400 mJ/cm$^2$ UVr dose, and for 18 seconds to achieve the 20 mJ/cm$^2$ UVr dose. Non-irradiated age-matched mice used as control were handled in the same fashion as the irradiated animals. Each group of animals was killed at different times after irradiation as described in each section. The mice were killed using a $CO_2$ gas chamber, and different tissues were removed according to each section. Procedures concerning animals followed the research animal use guidelines established by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET-Argentina) and approved by the Board of Ethical Review of the Instituto de Estudios de la Inmunidad Humoral (IDEHU).

### *Flow cytometric analysis*

Inguinal lymph nodes and spleens from UV-irradiated or control mice were manually dispersed with a tissue

homogenizer (Thomas Scientific, Swedesboro, NJ) and cells were counted. The following anti-mouse antibodies were purchased from BD Biosciences (San Jose, CA, USA): fluorescein isothiocyanate-conjugated anti-CD3ε; Alexa Fluor 647-conjugated anti-CD4; phycoerythrin-conjugated anti-CD8, phycoerythrin-conjugated anti-T-cell receptor γδ (TCR-γδ) with their corresponding isotype controls. For staining of surface markers, lymph node cells were incubated with antibodies diluted in staining buffer [phosphate-buffered saline (PBS), 10% fetal calf serum] for 30 min at 4°, washed, and then fixed in 0·2 ml of 2% formaldehyde (in PBS). Data were acquired on a PAS III cytometer (PARTEC, Münster, Germany) and analysed using CYFLOGIC software 1.2.1 (CyFlo Ltd, Turku, Finland). Dead T cells were excluded from flow cytometric analysis based on their forward-/side-scatter profile.

### *In vitro T-cell proliferation*

To perform T-cell proliferation assay, inguinal lymph nodes and spleen cell suspensions were placed in replicates of three ($4 \times 10^5$ cells in each well), together with 100 µl supplemented RPMI-1640 (with 10% fetal calf serum, streptomycin 100 µg/ml and penicillin 100 U/ml). Cells were incubated with 1 µg/ml of the non-specific T-cell mitogen concanavalin A (ConA) (Sigma Chemical Co., St Louis, MO). A basal proliferation control was also performed without adding mitogen. After 72 hr of incubation at 37° with 5% $CO_2$, supernatants were collected and MTT reagent (ThermoFisher Scientific, Carlsbad, CA) was used to measure cell proliferation. This was expressed as proliferation index (PI), which is defined as the ratio between stimulated and basal cell culture proliferation ($PI = OD_{540\ nm(x\ \mu g/ml\ ConA)}/OD_{540\ nm\ basal\ proliferation}$).

### *Cytokine quantification in culture supernatants*

Interleukin-4 (IL-4), IL-10 and interferon-γ (IFN-γ) levels were measured by ELISA using the OPTEIA system (BD Biosciences) according to the manufacturer's instructions in cell-free culture supernatants of the cells treated with 1 µg/ml ConA.

### *In vivo T-cell response; CHS response*

The CHS assay was performed using oxazolone diluted in ethanol (OXA; Sigma Chemical Company) as previously described.[18,19] The mice were irradiated with their ears covered to avoid UV irradiation. Twenty-four hours or 8 days after the single or the last exposure, the mice were sensitized by painting onto the abdomen 150 µl of 3% OXA. Six days later, the baseline ear thickness was measured with a micrometer (MG13180, Beijing, China). Then, a CHS response was elicited by painting the dorsal and ventral ear surfaces with 10 µl of 1% OXA. After 24 hr, the ear thickness was measured in a blinded manner. In all experiments, some mice were challenged but not sensitized with 3% OXA. The change in ear thickness was calculated as ΔT = (ear thickness 24 hr after elicitation) – (baseline ear thickness). The percentage of CHS suppression was calculated as % suppression = $(1–\Delta T_E/\Delta T_S) \times 100$, where $\Delta T_S$ is the change in ear thickness of the sensitized animal and $\Delta T_E$ is the change in ear thickness of animals exposed to experimental treatment.

### *Tetanus toxoid immunization scheme*

Twenty-four hours or 8 days after the single or the last UV exposure, the mice were bled to obtain pre-immune sera. Then, the mice were immunized subcutaneously on the left flank with 200 µl of tetanus toxoid commercial vaccine (Tetanol-Pur®; GlaxoSmithKline Argentina, Victoria, Buenos Aires, Argentina) diluted 1 : 2·5 in PBS. The same procedure was performed with sham-irradiated mice. Three weeks later, the mice were bled to death and serum samples were stored at −20° until serological testing.

### *Serum antibody titres*

Tetanus-toxoid-specific IgM, IgA, total IgG and IgG subisotypes IgG1, IgG2a, IgG2b and IgG3 were measured by ELISA in sera. Briefly, 96-well plates (Costar) were coated with 100 µl of tetanus toxoid (10 µg/ml in PBS) (Instituto Biológico de La Plata, Buenos Aires, Argentina) overnight at 4°. The plates were blocked with BSA-buffer (1% bovine serum albumin in PBS) for 1 hr at 37°. Then, the plates were washed with PBS-Tween 0·05% (PBST) and the serum samples collected from mice were serially diluted in BSA-buffer, in a 1 : 2 ratio, and 100 µl was added to the plates. Samples were incubated for 1 hr at 37°. The plates were washed with PBST and then incubated with 100 µl of peroxidase-labelled goat anti-mouse IgM, IgA, total IgG, IgG1, IgG2a, IgG2b or IgG3 (1 : 5000 in BSA-buffer) for 1 hr at 37°. The plates were washed again and the reaction was evidenced by the addition of TMB (3,3′,5,5′-tetramethylbenzidine) Chromogen Solution (for ELISA) (ThermoFisher Scientific) to each well. The TMB reaction was stopped with 2 M $H_2SO_4$, and the absorbance at 450 nm was read in an ELISA plate reader (Multishank-EX; ThermoFisher Scientific).

### *Staphylococcus aureus infection*

*Bacterial growth. Staphylococcus aureus* strain USA300-LAC was grown on tryptone soy broth at 37° with agitation (200 rpm) until an $OD_{600}$ of 0·8. Bacteria were harvested by centrifugation at 10·000 ***g*** for 10 min and cells


 

were washed twice with sterile PBS and resuspended to a final concentration of $1 \times 10^7$ colony-forming units (CFU)/ml. To obtain heat-killed bacteria, the bacterial suspension was heated for 2 hr at 90°.

*Skin infection model.* Mice were irradiated or mock-irradiated according to their specific irradiation protocol, and 24 hr after the single or the last UV exposure, mice were weighed and subcutaneously inoculated in the right side of the flank with $1 \times 10^7$ CFU/ml of *S. aureus*. Non-irradiated and non-infected mice were used as the infection control group. The progression of disease (skin lesion development) was monitored by daily measurement of the animal weight and the lesion dimensions [length (L) and width (W)] using a caliper. The lesion L and W were used to calculate the skin lesion area [$A = \pi \times (L/2) \times (W/2)$], as previously reported by Malachowa *et al*[17] Six days after infection, mice were killed and bled, and abscesses, spleen and lungs were removed from each animal. Blood samples were used to determine IgM- and IgG-specific antibody levels. Abscesses, lungs and spleens were homogenized and bacterial recovery was quantified in all of them. Cytokine levels were quantified in the abscess homogenates. Spleens were also used to analyse specific T-cell response.

*Specific T-cell response: spleen cell cultures.* Aseptically removed spleens were manually dispersed with a tissue homogenizer (Thomas Scientific, Swedesboro, NJ). Cells were centrifuged (10 min, 200 ***g***, 4°) and pellets were washed with sterile PBS. After the red blood cell lysis, cells were counted in a Neubauer's haemocytometer by trypan dye blue exclusion. One hundred microlitres of spleen cell suspensions ($4{\cdot}0 \times 10^6$ cells/ml) were cultured with the same volume of RPMI-1640 alone or with heat-killed bacteria ($2 \times 10^8$ CFU/ml) in triplicates at 37° and 5% $CO_2$ in air. ConA (1 μg/ml, 100 μl; Sigma) was used as a proliferation positive control. In each experiment, two cell cultures were performed: one to assess cell proliferation and the other to determine the cytokine levels in supernatants. After 72 hr, supernatants were collected and stored at −80° until assayed for cytokine levels. Proliferative responses were assessed by [$^3$H]thymidine (Perkin Elmer Inc., Waltham, MA, USA) uptake employing a Liquid Scintillation Analyzer 1600TR (CANBERRA PACKARD Central Europe GmbH, SCHWADORF, Austria). Proliferation results were expressed as mean counts/min ± SEM of triplicate cultures. BD OPTEIA ELISA kits (BD Biosciences) were employed to measure IL-4, IL-10 and IFN-$\gamma$ levels in cell-free culture supernatants as previously described.

*Specific B-cell response.* Specific IgM and IgG antibody levels were measured by indirect ELISA. Briefly, heat-killed bacteria were used as coating antigen at $5 \times 10^8$ CFU/ml in carbonate buffer: 15 mM $Na_2CO_3$; 3 mM $NaHCO_3$; pH 9·6. Antibodies were detected using a horseradish peroxidase-conjugated anti-mouse IgM or IgG (Bethyl Laboratories Inc., Montgomery, TX) with TMB Chromogen Solution and absorbance at 450 nm was read in an ELISA plate reader (Multishank-EX; Thermo Scientific).

*Statistical analysis*

All the values are presented as the mean ± SD. The statistical significance was evaluated using one-way analysis of variance (ANOVA). When the variables had a normal distribution and showed homoscedasticity, a parametric ANOVA and a Student–Newman–Keuls post-test were used. When samples did not have a normal distribution within any group or the experimental groups showed heteroscedasticity, a non-parametric ANOVA and a Dunn's post-test were used. For contingency analysis, Fisher Exact test was applied. Graphical and statistical analyses were performed with GraphPad Prism 5·0 (GraphPadSoftware, La Jolla, CA) and GraphPadInstat 2·0 (GraphPad Software), respectively. The values were considered significantly different at $P < 0{\cdot}05$, $n = 5$.

## Results

Skin exposure to an shUVd and rlUVd produces changes in the innate immune system as we have previously reported.[16] Based on those results, we have decided to evaluate the possible effects of UVr on adaptive immunity. We first analysed T-cell populations on draining lymph nodes, as a regional report of the changes initiated in the skin; afterwards, we looked into the same cell populations in the spleen to analyse systemic changes produced by UVr.

### T-cell populations in secondary lymphoid organs are differentially affected by shUVd and rlUVd irradiation

Skin exposure to an shUVd produced an increase of the percentage of T lymphocytes at the expense of $CD3^+$ $CD4^+$ cells in inguinal lymph nodes 24 hr post-UV, which persisted at day 8 after the irradiation (Figure 1a). No changes in the percentages of $CD3^+$ $CD8^+$ cells or TCR-$\gamma\delta^+$ cells were found at any time evaluated (Figure 1a). On the other hand, an shUVd did not significantly modify T-cell subpopulation percentages in the spleen at any time, but produced an increase of T lymphocytes ($CD3^+$ cells) percentage 24 hr post-UV and of the organ total cell number 8 days post-UV (Figure 1b). When analysing the effects produced by rlUVd, we observed lymph node hyperplasia 24 hr and 8 days post-UV, which correlates with the increments in T lymphocyte

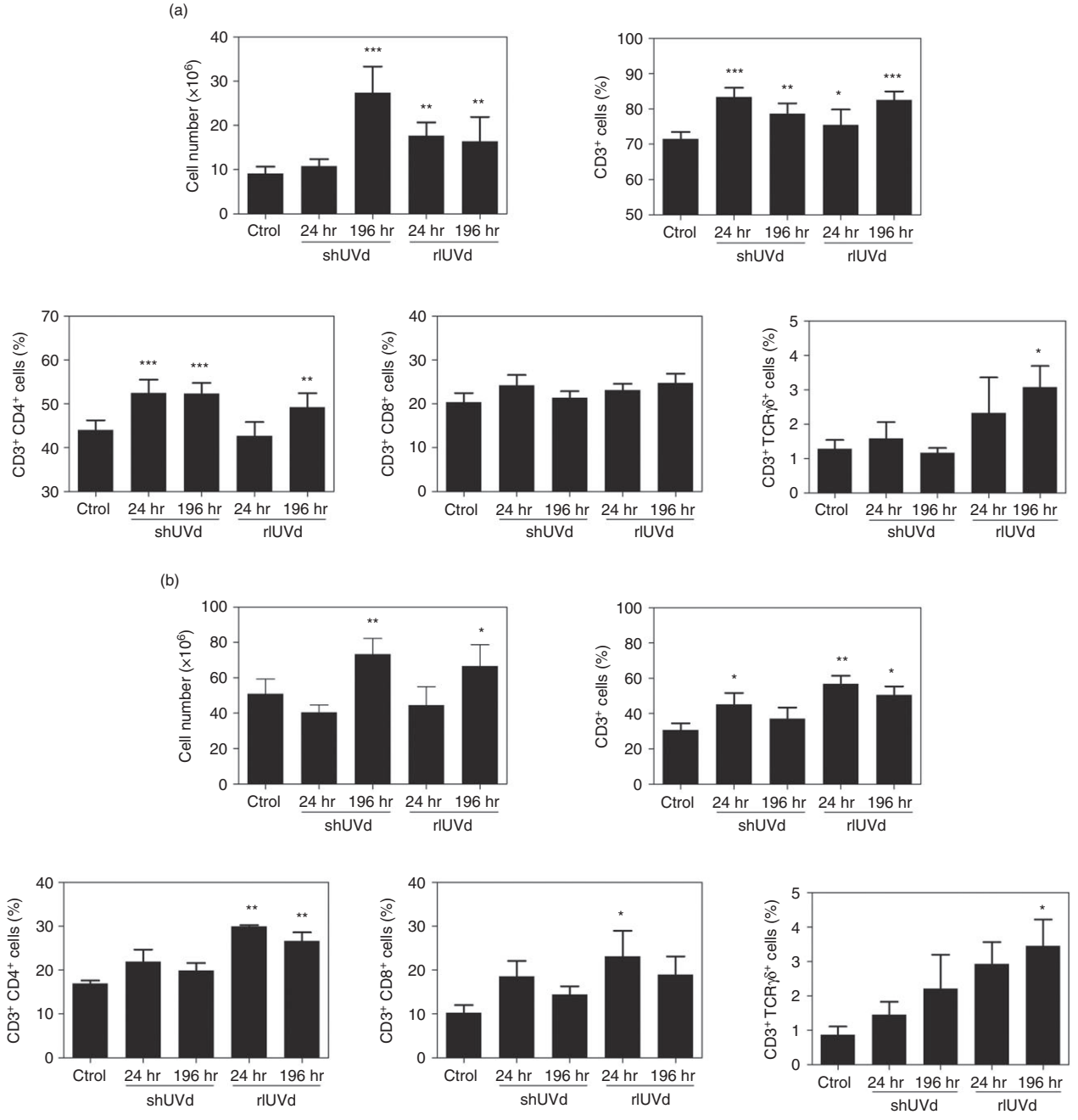


**Figure 1.** Inguinal lymph nodes (a) and spleen (b) total cell number and T-cell populations detected at different times (24 hr and 196 hr) after skin exposure to a single high UV dose (shUVd) and to repetitive low UV doses (rlUVd), and in the non-irradiated control group. The figures show the percentage of $CD3^+$, $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$ and $CD3^+$ TCR-$\gamma\delta^+$ cells. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$; $n = 5$. The results are expressed as mean ± SD.

percentages, meanwhile the percentages of $CD3^+$ $CD4^+$ and TCR-$\gamma\delta^+$ cells were increased (Figure 1a). In contrast to the effects observed by an shUVd in spleen, rlUVd produced a rapid increase (24 hr) of T lymphocytes at the expense of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ cells. Eight days after irradiation, $CD3^+$ $CD4^+$ cells were still increased in the spleen as well as TCR-$\gamma\delta^+$ cells, whereas $CD3^+$ $CD8^+$ cells returned to basal percentages (Figure 1b).

### shUVd alters T-cell response in skin-draining lymph nodes and spleen, but rlUVd only affects splenic T cells

No changes in T-cell proliferative response to ConA stimulation was observed in lymph node cells of mice exposed to an shUVd or rlUVd compared with control group. However, a great increase in IFN-$\gamma$ production was found in culture supernatant of ConA-stimulated lymph node cells from mice exposed to an shUVd at both evaluated times


 

(Figure 2a). On the other hand, when we looked into the spleen T-cell response, mice exposed to both an shUVd and an rlUVd diminished ConA-induced proliferation 24 hr after UV. Repetitive low UVd also decreased T-cell proliferation 8 days after UV exposure. Despite this effect, no significant differences in cytokine production were found among groups at any time evaluated (Figure 2b).

### shUVd suppresses specific T-cell responses *in vivo*, whereas rlUVd stimulates it

To investigate the role of UVr in the modulation of T-cell function, we sensitized mice with OXA in the abdomen (non-irradiated skin) 24 hr or 8 days after the last or the single irradiation. Five days later we challenged the animals, to evaluate specific T-cell response induced. As it has been extensively reported during the last four decades, skin exposure to an shUVd diminishes CHS reaction to OXA if the sensitization occurred 24 hr after the irradiation (37% of immunosuppression). When OXA was applied to mouse abdomens 8 days post-UV, no changes in CHS response were seen compared with the control group. Surprisingly, rlUVd enhanced the CHS response when OXA was applied 24 hr and 8 days after mouse UV exposure (42% and 48% of immunostimulation, respectively) (Figure 2c).

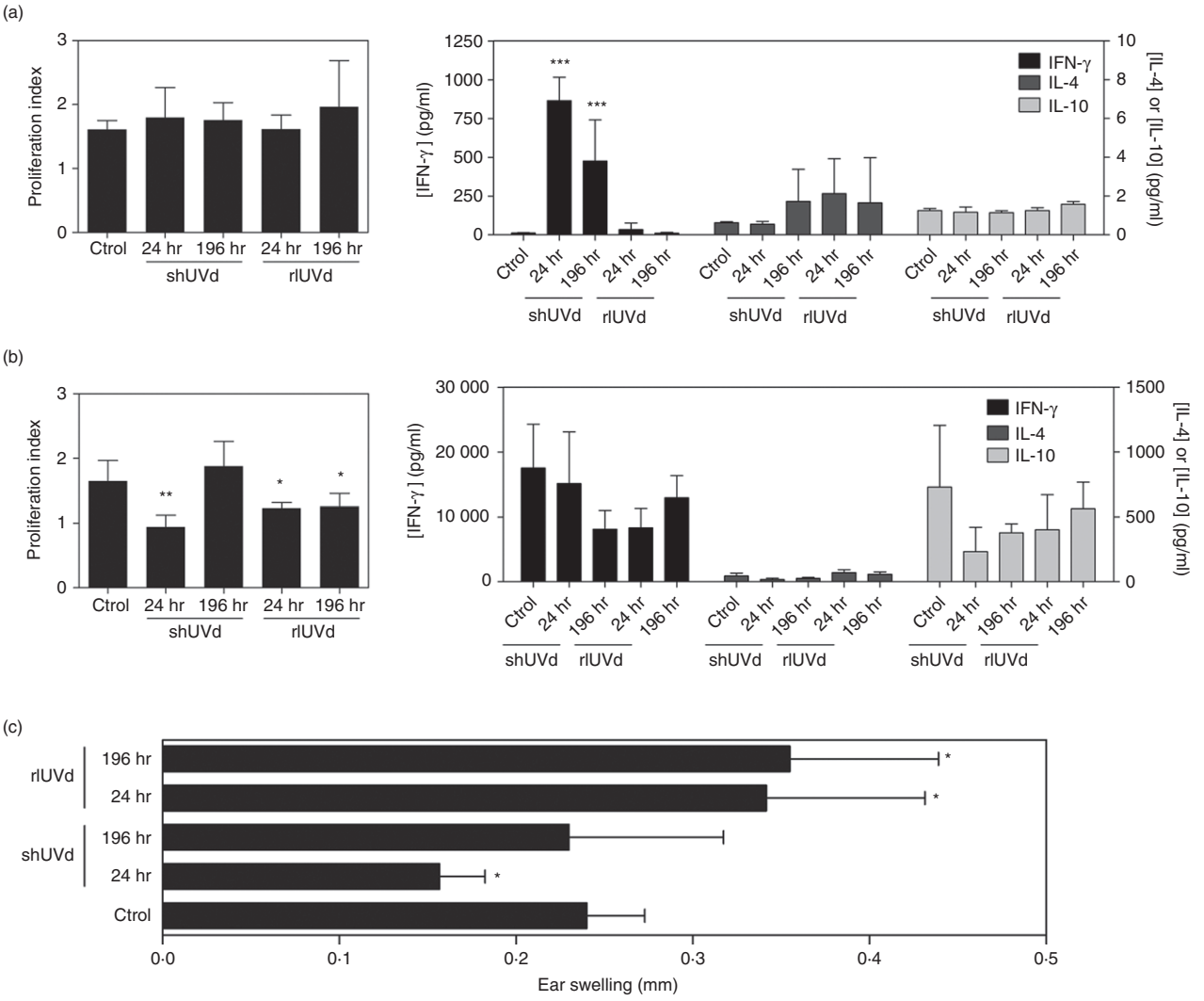


**Figure 2.** T-cell function evaluated *in vitro* by unspecific stimulation and *in vivo* by contact hypersensitivity (CHS) reaction. Concanavalin A stimulation of inguinal lymph nodes (a) and spleen (b) cells after skin exposure to a single high UV dose (shUVd) and to repetitive low UV doses (rlUVd), and in a non-irradiated control group. The values are expressed as proliferation index (PI). PI is defined as the ratio between stimulated and basal cell culture proliferation ($PI = OD_{540\ nm(x\ \mu g/ml\ ConA)}/OD_{540\ nm\ basal\ proliferation}$). Interferon-$\gamma$ (IFN-$\gamma$), interleukin-10 (IL-10) and IL-4 levels in culture supernatant after inguinal lymph nodes (a) and spleen (b) cell unspecific stimulation is shown. The values are expressed as pg/ml. (c) Contact hypersensitivity (CHS) reaction performed 24 hr and 8 days after skin exposure to an shUVd and rlUVd, and in non-irradiated control group. Results are expressed as ear swelling in mm. Ear swelling represents the difference between the ear thickness 24 hr after elicitation and the baseline ear thickness. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$; $n = 6$. The results are expressed as mean ± SD.

### An shUVd diminishes specific antibody production, while rlUVd increases it

To further investigate the role of UVr in the modulation of B-cell function, we immunized mice with alum-conjugated tetanus toxoid vaccine 24 hr or 8 days after the last or the single irradiation, to evaluate specific antibody production in serum. Skin exposure to an shUVd decreased specific anti-tetanus toxoid total IgG and IgG2b levels when mice were immunized 24 hr post-UV. However, if the immunization occurred 8 days after mouse exposure, the titre of these antibodies did not change compared with the control group. An shUVd also produced a slight increase of specific IgA when mice were immunized both 24 hr and 8 days post-UV. In contrast to these results, mice immunized 24 hr after skin exposure to rlUVd, increased total IgG and IgG3 titres, but when immunization occurred 8 days post-UV, only a slight induction of IgA was observed. No differences in IgM, IgG1 and IgG2a serum levels were observed among groups (Figure 3).

### rlUVd leads to a defective control of *S. aureus* cutaneous infection

As previously reported, UVr is able to perturb the skin barrier function.[16,14] Moreover, as shown here, UVr can also modulate the adaptive immune system, depending on the dose of radiation received. Consequently, we decided to investigate how UVr is able to modulate the immune response against a complex challenge, such as a living pathogen. Therefore, we infected UV-irradiated and mock-irradiated mice with *S. aureus* (USA300-LAC), as this bacterium is responsible for most of the skin and soft-tissue infections.

The infection was monitored by a daily measurement of mouse weight and abscess size. The main weight loss occurred at 24 hr post-inoculation in all mice (Figure 4a). We observed that animals exposed to an shUVd experienced a marked weight drop compared with those exposed to rlUVd (Figure 4b). Besides this, all mice were able to develop skin abscesses, but no differences in area size were measured (Figure 4c). Then, we evaluated

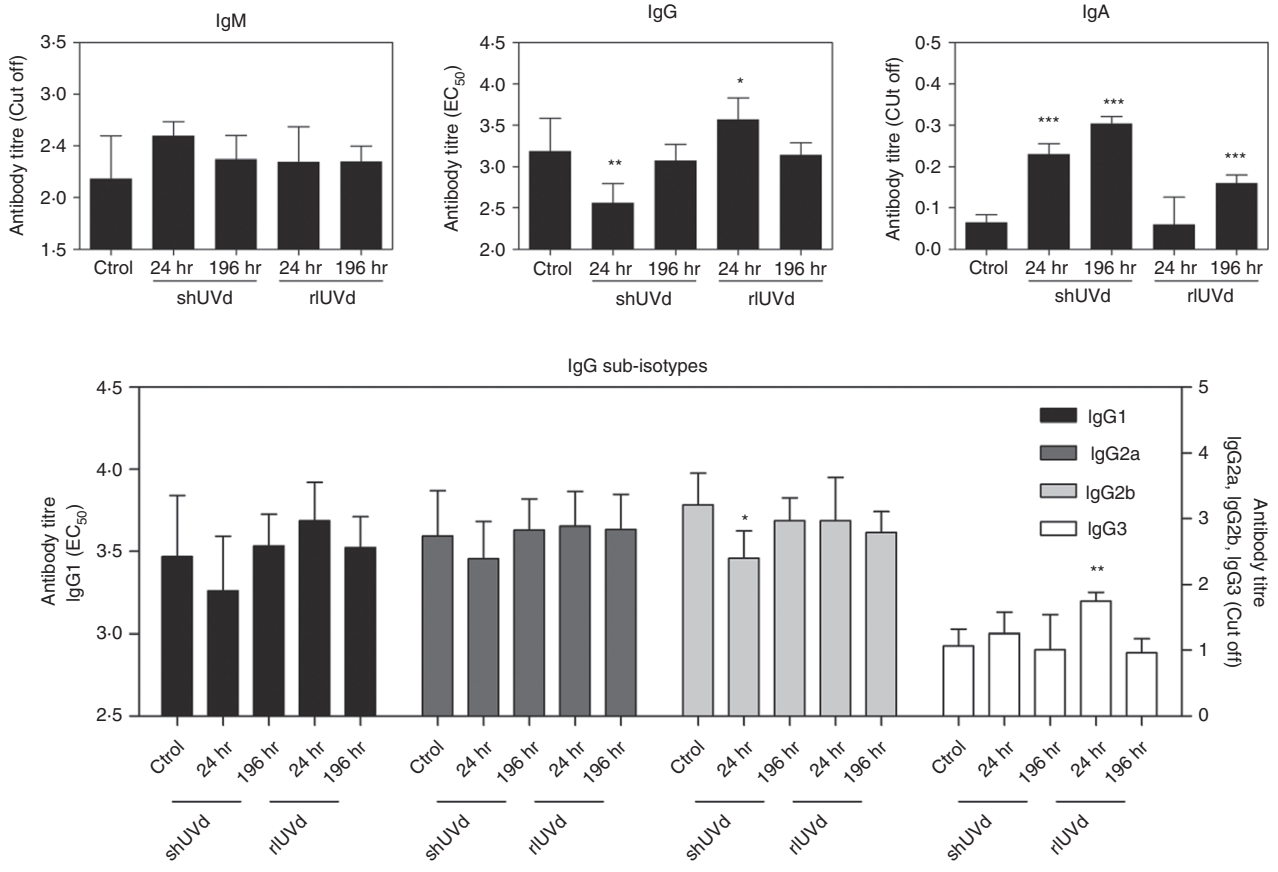


**Figure 3.** Anti-tetanus toxoid (TT) specific antibody production. Mice were subcutaneously immunized with TT 24 hr or 196 hr after skin exposure to a single high UV dose (shUVd) and repetitive low UV doses (rlUVd), and in non-irradiated control group. Three weeks later, specific IgM, IgG, IgA and IgG sub-isotypes were measured in sera. The titre was determined as EC50, when the titration curves were complete, or as a cut-off, when the titration curves were incomplete. The cut-off was obtained as the triple of the minimal signal (bottom $OD_{450\ nm} \times 3$). $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$; $n = 5$. The results are expressed as mean ± SD.


 

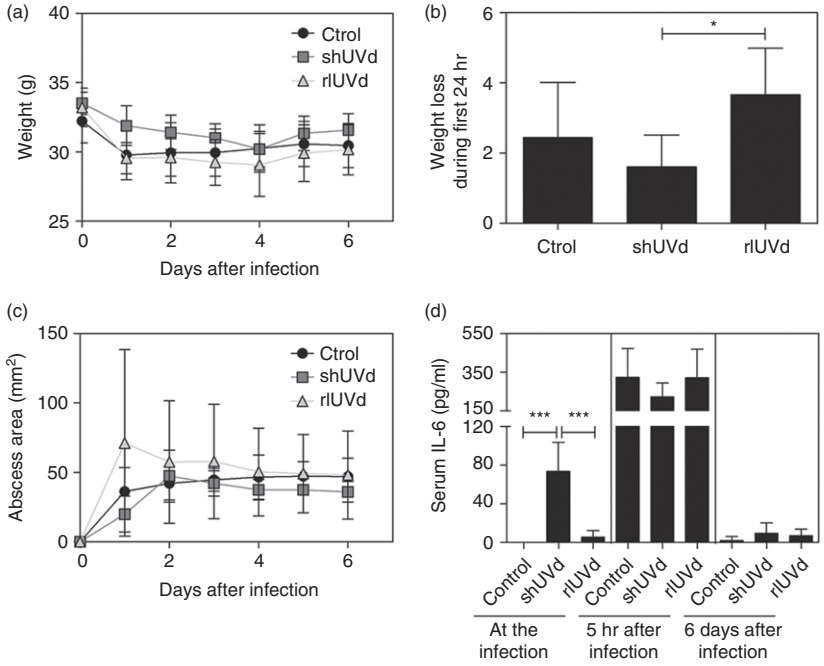


**Figure 4.** Daily evaluation of animal weight (a, b) and abscess size (c) during *Staphylococcus aureus* infection. Interleukin-6 (IL-6) levels were measured in sera before the inoculation, and 5 hr and 6 days post-inoculation (d). $*P < 0{\cdot}05$, $***P < 0{\cdot}001$; Control: $n = 7$; single high UV dose (shUVd): $n = 5$; repetitive low UV doses (rlUVd): $n = 6$. The results are expressed as mean ± SD.

serum IL-6 levels immediately before the inoculation, 5 hr and 6 days post-challenge. Mice exposed to an shUVd presented increased levels of IL-6 at the moment of inoculation. Five hours post-challenge, increased levels of IL-6 in serum were found in all the animals but no differences among groups were observed. At six days post-inoculation, IL-6 levels decreased in all groups (Figure 4d).

To continue with the evaluation of the local infection, we compared the size of the abscesses at the end of the experiment. Despite no differences in the final area size among groups (Figure 5a), the abscesses formed in rlUVd-exposed mice were heterogeneous and presented an invasive-like characteristic, as they were attached to the peritoneum (data not shown). After performing the bacterial count in the abscesses, we found no differences in bacterial recovery among groups (Figure 5a).

*Staphylococcus aureus* elimination requires neutrophil recruitment and abscess formation in response to the cytokines and chemokines released by keratinocytes, macrophages and other resident cells.[20–22] We observed a reduction in CXCL-1 and tumour necrosis factor-$\alpha$ level in abscess homogenates of shUVd-exposed mice compared with control and rlUVd groups (Figure 5b) at 6 days post-inoculation. No differences in IL-6 and IL-1$\beta$ levels were observed among groups.

To investigate if abscess formation was effective in controlling bacterial dissemination, we determined distal organ colonization by quantifying the bacteria present in the spleen and lungs of infected mice. rlUVd promoted *S. aureus* colonization of the spleen, as shown by an increase in the number of mice with bacteria in this organ (five animals colonized from a total of six inoculated) (Figure 6a). The same tendency was observed in lungs but the results were not statistically significant. Skin exposure to an shUVd did not modify the number of mice with spleen and lung colonized with the bacteria compared with the control group.

### rlUVd bias specific T helper response against *S. aureus* towards a T helper type 2 profile

Spleen cell suspensions from rlUVd-exposed mice significantly increased the T-cell proliferative response against *S. aureus* antigens, even though all groups of mice were able to respond to the bacterial stimuli (Figure 6b). The *S. aureus*-stimulated cells released high levels of IFN-$\gamma$ and IL-10 in all groups, with no differences in concentration among them. Interestingly, only splenocytes from mice exposed to rlUVd were able to produce IL-4 in response to *S. aureus* stimuli (Figure 6b). Finally, we looked into specific antibody production. Six days after inoculation, mice exposed to rlUVd presented high levels of IgM whereas an shUVd increased both IgM and IgG antibodies (Figure 6c) compared with the non-irradiated *S. aureus*-inoculated control group.

## Discussion

Human beings, according to the place of residence and the season, may be constantly exposed to solar radiation, as a result of being outdoors for short or long periods of time. The skin, as the outermost organ of the body, absorbs UVr and activates multiple mechanisms to maintain skin

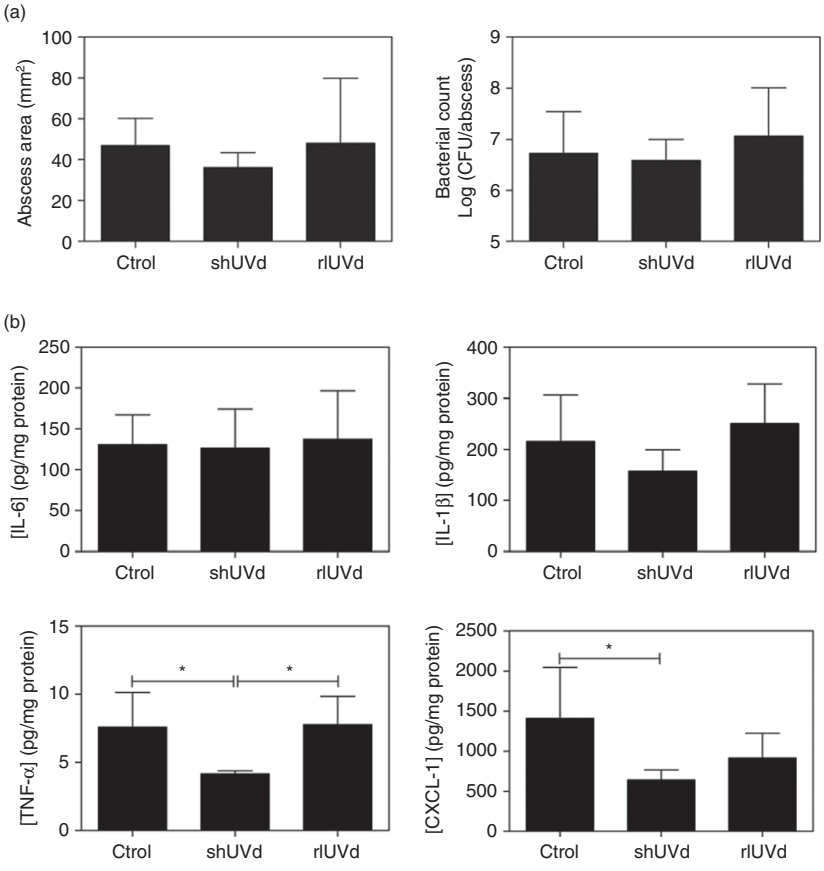


**Figure 5.** Evaluation of local infection and inflammatory response in irradiated and control *Staphylococcus aureus*-infected mice. Six days after *S. aureus* inoculation, the final abscess area and the bacterial count were determined (a). Tumour necrosis factor-$\alpha$ (TNF-$\alpha$), interleukin-6 (IL-6), IL1-$\beta$ and CXCL-1 levels were measured in abscess homogenates (b). Results are expressed as pg cytokine/mg protein. *$P < 0{\cdot}05$. Ctrol: $n = 7$; single high UV dose (shUVd): $n = 5$; repetitive low UV doses (rlUVd): $n = 6$. The results are expressed as mean $\pm$ SD.

homeostasis.[23,24] In 1978, Streilein defined the skin-associated lymphoid tissue as an integrated system in which Langerhans cells, keratinocytes, resident epidermal cells, migrating lymphocytes and draining lymph nodes all work together coordinately to provide the skin with immune protection.[25,26] Since then, the skin has been considered an immune organ and plenty of research has been performed in the field.[27,28] Based on our previous results comparing shUVd and rlUVd effects on skin innate immune system,[16] we decided to study if these changes can also modulate the adaptive immune response.

To initiate the study of UVr effects on adaptive immunity, we selected draining lymph nodes (inguinal lymph nodes) and spleen as secondary lymphoid organs that allow determination of the possible indirect effects observed after skin exposure to UVr, close to as well as distant from the irradiation site.

The results of this work show that 24 hr after skin exposure to an shUVd, the percentage of $CD3^+$ $CD4^+$ cells in inguinal lymph nodes is increased. As we previously reported, an shUVd produces a high inflammatory response in the site of irradiation. As a consequence of this inflammatory damage, Langerhans cells in the epidermis and dendritic cells in the dermis might be sensing altered cells (through damage-associated molecular patterns) or microorganisms (through pattern-associated molecular patterns) that were able to go through the epithelium, and might be migrating to the draining lymph nodes to activate the adaptive immune response. Even though no differences in T-cell proliferation were observed *in vitro*, higher amounts of IFN-$\gamma$ were quantified in culture supernatants, indicating a possible T helper type 1 differentiation. This T helper type 1 cells may collaborate with macrophages to eliminate damaged cells and microorganisms. Skin exposure to an shUVd produces a decrease in epidermal $\gamma\delta$ T-cell number. However, this effect does not seem to modify $\gamma\delta$ T-cell recirculation among lymphoid organs, as the percentage of these cells did not change in any of the organs studied at any time-point.

As previously reported,[29,30] an shUVd produces a transient reduction in CHS response to OXA when the sensitization phase occurs 24 hr post-UV. This result represents a 37% immunosuppression and correlates with the diminished proliferative T-cell response observed in splenic ConA-stimulated cells *in vitro*, 24 hr post-UV. These results are in concordance with the reduction in specific anti-tetanus toxoid IgG serum levels, observed when immunization was performed also 24 hr post-UV. Therefore, we are able to conclude that an shUVd modulates the immune system in different ways. On the one hand, this exposure produces an enhancement of innate


 

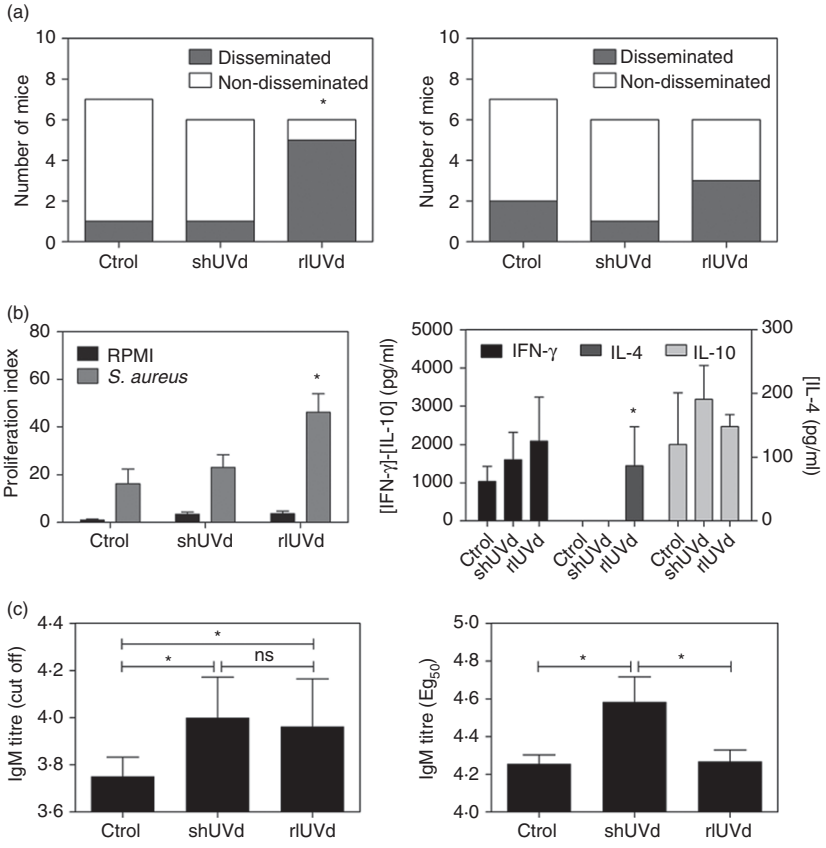


**Figure 6.** Evaluation of systemic infection and immune response in irradiated and control *Staphylococcus aureus*-infected mice. (a) Bacterial colonization into the spleen and lungs. The results are expressed as the number of mice with bacterial growth detected in the organ. (b) Specific spleen T-cell stimulation with heat-killed *S. aureus* and interferon-$\gamma$ (IFN-$\gamma$), interleukin-4 (IL-4) and IL-10 quantification in culture supernatant. (c) Specific IgM and IgG titre in serum, 6 days post-*S. aureus* inoculation. $*P < 0{\cdot}05$. Ctrol: $n = 7$; single high UV dose (shUVd): $n = 6$; repetitive low UV doses (rlUVd): $n = 6$. The results are expressed as mean $\pm$ SD.

immunity, as previously reported by us and other authors,[16,31–33] on the other hand, it promotes a systemic immunosuppression proved by a decrease in both specific T-cell and B-cell responses. This suppression of the immune response is evidenced when mice are challenged with isolated molecules such as OXA and TT.

Surprisingly, skin exposure to rlUVd enhances the mouse CHS response to OXA when the sensitization occurs at both 24 hr and 8 days post-UV, which corresponds to a 42% and 48% of immunostimulation, respectively. This potentiated specific cellular response may be a consequence of the spleen T-cell activation observed ($CD4^+$ and $\gamma\delta$ T cells). This 'immunostimulatory effect' of UVr on the cellular components of the immune system has not been previously reported, to the best of our knowledge. Opportunely, the specific B-cell response is also enhanced in mice exposed to rlUVd, evidenced by an increase in specific IgG titres in sera of irradiated mice. Interestingly, we found a sustained increase in $\gamma\delta$ T cells in skin-draining lymph nodes and spleen. We suggest that these $\gamma\delta$ T cells are recirculating through lymph nodes and the skin, as we previously reported an increase in CXCL-12 transcription in epidermis and dermis, which in turn resulted in a slight lymphoid infiltrate in dermis that may contribute to the maintenance of the $\gamma\delta$ T-cell pool in the epidermis.[16] Then, we are able to conclude that rlUVd potentiate adaptive immune responses with no effects on the innate immune system.

To further study these opposite effects observed after skin exposure to UVr, we decided to evaluate how UVr determines the global immune response during a bacterial infection. To achieve this aim, we chose *S. aureus* as the bacterial challenge, because this microorganism is capable of causing skin infections.[34–36] We decided to perform subcutaneous inoculation of *S. aureus* (USA300-LAC)[17] 24 hr after the single or the last UV exposure, as the main differences in the immune responses were observed at this time post-UV. The shUVd exposure results in a disruption of the epidermal barrier, with a concomitant inflammatory state characterized by the production of pro-inflammatory cytokines and chemokines, which in turn recruit innate immune cells to the irradiated area, such as neutrophils and monocytes. In contrast, 24 hr after skin exposure to rlUVd there is a reinforcement of the epidermal barrier, with no sign of inflammation, but with an increment in vascular endothelial growth factor-$\alpha$ and antimicrobial peptides transcription, as we previously described.

As a consequence of the shUVd-induced immunosuppression, an increased susceptibility to infections would be expected. However, exposure to this irradiation schedule before infection does not seem to affect the control of

the bacterial infection, as bacterial counts do not differ from control mice. Moreover, shUVd exposed animals were able to control the infection better than the rlUVd irradiated ones. A possible explanation for this might be the inflammatory response that was already established in the skin, which could lead to a better abscess formation, which in turn would prevent dissemination of bacteria to the lungs and spleen. Hence, when mice are subcutaneously exposed to a complex challenge (such as living *S. aureus*), the pre-existing activation of the innate immunity may be more relevant than the suppression of the adaptive immune system.

In contrast, rlUVd exacerbated the bacterial infection, as it promoted the colonization of the spleen. Hence, in spite of the reinforcement of the epithelium, if the bacteria penetrate the skin, the absence of inflammation and the enhanced dermal vascularization might contribute to the leakage of the bacteria. In this case, the adaptive immune stimulation observed is irrelevant in contrast to the lack of innate immune response.

Taking these findings together, we are able to conclude that skin exposure to UVr modulates the immune system in different ways depending on the dose received, and the presence and nature of a specific stimulus. An shUVd induces the innate immunity *per se* and suppresses the adaptive immune system when mice are challenged with simple molecules, such as OXA and tetanus toxoid. However, in the presence of more complex stimuli this immunosuppression becomes insignificant and the innate immunity becomes essential to protect skin from *S. aureus* infection. Then, this beneficial effect of UVr might be used as phototherapy to treat certain skin infections. On the other hand, rlUVd does not impact on innate inmunity *per se* but enhances both B-cell and T-cell responses when mice are challenged with those simple molecules. This improvement in tetanus toxoid vaccination as a consequence of UVr exposure must be further investigated, including other vaccines, to consider the exposure to certain UVr doses a possible enhancer to normal vaccination procedures.

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## Disclosures

None declared.

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