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Article type : Original Article

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

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/imm.12901

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Short title: Effects of daily vs. harmful UV exposures on the immune response

Keywords: immunesuppression · Ultraviolet radiation · Skin · bacterial infection · immunestimulation

Abstract

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 or 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 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 and B cell responses after exposure to a 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.

Introduction

Ultraviolet radiation (UVr), contained within sunlight that reaches the Earth 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 very 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, MAPKs 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-vapor lamp (9). Plenty of experimental conditions were analyzed since then in animal models; we would like to highlight the following concepts: 1) 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 a half MED) (10,11). 2) Acute exposure to UVr promotes a deficient adaptive immune response; this very 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 a half MED and applied on a few consecutive days). Cutaneous T cell response to different sensitizers (oxazolone, DNFB, etc.) is the most common technique employed to report the UV-induced immunosuppression

(12,13). 3) Exposure to UV light have 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) has shown to induce the production of antimicrobial peptides on the skin, probably through the synthesis of that vitamin (14). 4) 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 abovementioned 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 consisted in exposing the animals to a single high UV dose (shUVd, 2 MEDs), 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 it has been very well described before, that exposure to a shUVd promoted local inflammation (by the production / by inducing pro-inflammatory cytokines and chemokines both in epidermis and dermis and recruiting neutrophils and lymphocytes), epidermal damage and a slight increase in anti-microbial peptides, without affecting vascularization of the skin. On the other hand, exposure to rlUVd was unable to induce any sign of local inflammation but it promoted a marked increase in anti-microbial peptides transcription and in vascularization (by increasing the transcription of VEGF in epidermis and dermis) while it promoted a slight epidermal hyperplasia. We have proposed that rlUVd promotes a reinforcement of the barrier function of the skin, without generating deleterious effects on the tissue.

Considering the opposite 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 on skin draining lymph nodes and spleen of animals exposed to shUVd and rlUVd using *in vitro* assays. Then, specific *in*

vivo T and B cell responses were analyzed through 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 (20-25g), purchased from Charles River Laboratories, were housed in a 12/12-hour light/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, USA), 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 an UVX radiometer (UVP).

The mice were irradiated on their backs with a single high dose of UVr (400 mJ/cm^2, corresponding to 2 MEDs) simulating a harmful exposure, or with 4 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 during 6 minutes and 4 seconds to achieve the 400 mJ/cm^2 UVr dose, and during 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 sacrificed at different times after irradiation as it is described in each section. The mice were sacrificed 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: FITC-anti-CD3ε; Alexa Fluor 647-anti-CD4; PE-anti-CD8, PE-anti-γδ TCR with their corresponding isotype controls. For staining of surface markers, lymph nodes cells were incubated with antibodies diluted in staining buffer (PBS, 10% FCS) for 30 minutes at 4°C, washed, and then fixed in 0.2 ml of 2% formaldehyde (in PBS). Data was acquired on a PAS III cytometer (PARTEC, Münster, Germany) and analyzed using Cyflogic software 1.2.1 (CyFlo ltd., Turku, Finland). Dead T cells were excluded in flow cytometric analysis based on their FSC/SSC profile.

***In vitro* T Cell Proliferation**

To perform T cell proliferation assay, inguinal lymph nodes and spleen cell suspensions were placed in replicates of 3 (4×10^5 cells in each well), together with 100 µl supplemented RPMI (with 10% fetal calf serum [FCS], streptomycin 100 µg/ml, and penicillin 100 U/ml). Cells were incubated with 1 µg/ml of the nonspecific T-cell mitogen concanavalin A (ConA) (Sigma Chemical Co., St. Louis, MO). A basal proliferation control was also performed without adding mitogen. After 72 hours of incubation at 37°C with 5% CO_2, supernatants were collected and MTT reagent (Thermo Fisher Scientific, Carlsbad, CA) was used to measure cell proliferation, which is expressed as proliferation index (PI). PI is defined as the

ratio between stimulated and basal cell culture proliferation (PI = $OD_{540nm(X\ \mu g/ml\ ConA)}$/ $OD_{540nm\ basal\ proliferation}$).

Cytokine quantification in culture supernatants

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

***In vivo* T Cell Response. Contact Hypersensitivity (CHS) Response**

CHS assay was performed using Oxazolone diluted in ethanol (OXA, Sigma Chemical Company, St Louis, MO) as previously described (18,19). The mice were irradiated with their ears covered in order 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 dorsal and ventral ear surfaces with 10 μl of 1% OXA. After 24 hours, 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 24hours after elicitation) – (baseline ear thickness).** The percentage of CHS suppression was calculated as **% suppression = (1 – ΔT_E / ΔT_S) x 100**, where ΔT_S is "change in ear thickness" of sensitized animal and ΔT_E is "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 in order 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 sera samples were stored at -20 °C until serological testing.

Serum antibody titers

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 37 °C. The plates were blocked with BSA-buffer (1% BSA in Phosphate Buffered Saline (PBS)) for 1 hour at 37 °C. 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 then 100 µl were added into the plates. Samples were incubated for 1 hour at 37 °C. The plates were washed with PBST and then incubated with 100 µl of peroxidase-labeled goat anti-mouse IgM, IgA, total IgG, IgG1, IgG2a, IgG2b or IgG3 (1:5000 in BSA-buffer) for 1 hour at 37 °C. The plates were washed again and the reaction was evidenced by the addition of TMB Chromogen Solution (for ELISA) (ThermoFisher Scientific) to each well. The TMB reaction was stopped with 4 N 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

S. aureus strain USA300-LAC was grown on tryptone soy broth (TSB) at 37°C 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 $1x10^7$ UFC/ml. To obtain heat-killed bacteria, the bacterial suspension was heated for 2 hours at 90°C.

Skin infection model

Mice were irradiated or mock-irradiated according to their specific irradiation protocol, and 24 hours after the single or the last UV-exposure, mice were weighted and subcutaneously inoculated in the right side of the abdomen with $1x10^7$ UFC/ml of *S. aureus*. Non-irradiated and non-infected mice were used as 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 it was previously reported by Malachowa, *et al* (17). Six days post-infection, mice were sacrificed and bled, and abscesses, spleen and lungs were removed from each animal. Blood samples were used to determine IgM and IgG specific antibodies 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 analyze 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 minutes, 200 *g*, 4°C) 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 (4.0×10^6 cells/mL) were cultured with the same volume of RPMI alone or with heat-killed bacteria ($2x10^8$ UFC/ml) in triplicates at 37°C and 5% CO_2 in air. Concanavalin A (1μg/mL, 100 μL; Sigma Chemical Co., St. Louis, MO) 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 hours, supernatants were collected and stored at -80°C until assayed for cytokine levels. Proliferative responses were assessed by [^{3}H]-thymidine (PerkinElmer Inc., USA) uptake employing a Liquid Scintillation Analyzer 1600TR (Packard, USA). Proliferation results were expressed as mean counts per minute (cpm) ± SEM of triplicate cultures. BD OPTEIA ELISA kits (BD Biosciences, Franklin Lakes, NJ, USA) were employed to measure IL-4, IL-10 and IFN-γ 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 $5x10^8$ UFC/ml in Carbonate Buffer: 15 mM Na_2CO_3; 3 mM $NaHCO_3$; pH: 9,6. Antibodies were detected using an HRP-conjugated anti-mouse IgM or IgG (Bethyl Laboratories Inc) 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, USA) and GraphPadInstat 2.0 (GraphPad Software), respectively. The values were considered different at $p<0.05$, 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 analyzed 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 analyze 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 hours post-UV, which persisted at day 8 after the irradiation (Figure 1 a). 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 hours post-UV and of the organ total cell number 8 days post-UV (Figure 1b). When analyzing the effects produced by rlUVd, we observed lymph nodes hyperplasia 24 hours and 8 days post-UV that correlates with the increments in T lymphocytes percentage, 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 hours) 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, while $CD3^+CD8^+$ 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 nodes cells of mice exposed to an shUVd or rlUVd compared to control group. However, a great increase in IFN-γ production was found in culture supernatant of ConA-stimulated lymph nodes cells from mice exposed to an shUVd at both evaluated times (Figure 2a). On the other hand, when we looked into spleen T cell response, mice exposed to both an shUVd and rlUVd diminished ConA-induced proliferation 24 hours post-UV. rlUVd 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*, while 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 hours 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 hours after the irradiation (37% of immunosuppression). When OXA was applied on mice abdomens 8 days post-UV, no changes in CHS response was evidenced compared to the control group. Surprisingly, rlUVd enhanced CHS response when OXA was applied 24 hours and 8 days after mice UV exposure (42 and 48% of immunostimulation, respectively) (Figure 2c).

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 (TT) vaccine 24 hours or 8 days after the last or the single irradiation, in order to evaluate specific antibodies production in serum. Skin exposure to an shUVd decreased specific anti-TT total IgG and IgG2b levels when mice were immunized 24 hours post-UV. However, if the immunization occurred 8 days after mice exposure, the titer of these antibodies did not change compared to control group. An shUVd also produced a slight increase of specific IgA when mice were immunized both 24 hours and 8 days post-UV. In contrast to these results, mice immunized 24 hours after skin exposure to rlUVd, increased total IgG and IgG3 titers, but when immunization occurred 8 days post-UV, only a slight induction of IgA was observed. No differences in IgM, IgG1 and IgG2a sera levels were observed among groups (Figure 3).

rlUVd leads to a defective control of *Staphylococcus aureus* cutaneous infection

As it has been previously reported, UVr is able to perturb the skin barrier function (16)(14). Moreover, as it is showed in this work, 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 measure of mice weight and abscess size. The main weight loss occurred at 24 hours post-inoculation in all mice (Figure 4a). We observed that animals exposed to an shUVd experienced a marked weight drop compared to those exposed to rlUVd (Figure 4b). Besides this, all mice were able to develop skin abscess, but no differences in area size were measured (Figure 4c). Then, we evaluated serum IL-6 levels immediately before the inoculation, 5 hours 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 were measured among groups (Figure 5a), the abscesses formed in rlUVd-exposed mice were very 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).

S. aureus elimination requires neutrophil recruitment and abscess formation in response to cytokine and chemokine released by keratinocytes, macrophages and other resident cells (20–22). We observed a reduction in CXCL-1 and TNF-α level in abscesses homogenates of shUVd-exposed mice compared to control and rlUVd groups (Figure 5b) at 6 days post-inoculation. No differences in IL-6 and IL-1β levels were observed among groups.

In order 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, evidenced by an increase in the number of mice with bacteria in this organ (5 animals colonized from a total of 6 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 to the control group.

rlUVd bias specific Th response against *S. aureus* towards a Th2 profile

Spleen cell suspensions from rlUVd-exposed mice significantly increased 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-γ 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 post-inoculation, mice exposed to rlUVd presented high levels of IgM while an shUVd increased both IgM and IgG antibodies (figure 6c) compared to 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 in order to maintain skin 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 which allow to determine the possible indirect effects observed after skin exposure to UVr, near as well as distant from the irradiation site.

The results of this work show that 24 hours 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 dentritic cells in the dermis might be sensing altered cells (through DAMPs) or microorganisms (through PAMPs) that were able to go through the epithelium, and might be migrating to the draining lymph nodes in order to activate the adaptive immune response. Even though no differences in T cell proliferation were observed *in vitro*, higher amounts of IFN-γ were quantified in culture supernatants, indicating a possible Th1 differentiation. This Th1 cells may collaborate with macrophages to eliminate damaged cells and microorganisms. Skin exposure to an shUVd

produces a decrease in epidermal γδ T cell number. However, this effect does not seem to modify γδ 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 it has been previously reported (29,30), an shUVd produces a transient reduction in CHS response to OXA when the sensitization phase occurred 24 hours post-UV. This result represents a 37% of immunosuppression and correlates with the diminished proliferative T cell response observed in splenic ConA-stimulated cells *in vitro*, 24 hours post-UV. These results are in concordance with the reduction in specific anti-TT IgG sera levels, observed when immunization was performed also 24 hours post-UV. Therefore, we are able to conclude that an shUVd modulates the immune system in different ways. On one hand, this exposure produces an enhancement of innate 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 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 mice CHS response to OXA when the sensitization occurs at both 24 hours 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 γδ 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, specific B cell response is also enhanced in mice exposed to rlUVd, evidenced by an increase in specific IgG titers in sera of irradiated mice. Interestingly, we found a sustained increase in γδ T cells in skin drainig lymph nodes and spleen. We suggest that these γδ 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 γδ T pool in the epidermis (16). Then, we are able to conclude that rlUVd potentiate adaptive immune responses with no effects on innate immune system.

In order 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, as this microorganism is capable of causing skin infections (34–36). We decided to perform subcutaneous inoculation of *S. aureus* (USA300-LAC) (17) 24 hours after the single or the last UV-exposure, as the main differences in the immune responses were observed at this time post-UV. 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 hours after skin exposure to rlUVd there is a reinforcement of the epidermal barrier, with no sign of inflammation, but with an increment in VEGF-α 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 prior to infection does not seem to affect the control of the bacterial infection, since 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 turns would prevent dissemination of bacteria to the lungs and spleen. Thus, when mice are subcutaneously exposed to a complex challenge (such as living

S. aureus), the pre-existing activation of the innate immunity may result more relevant than the suppression of the adaptive immune system.

On the other hand, 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.

Taken 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 turns 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 kind of skin infections. On the other hand, rlUVd do not impact on innate inmunity *per se* but enhance both, B 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, in order to consider the exposure to certain UVr doses a possible enhancer to normal vaccination procedures.

Acknowledgements

This work was supported by the University of Buenos Aires (UBACyT 2011-2014, CONICET PIP 2011-2013 and PIP 2014-2016 Grants). The authors would also like to thank Ms Carolina Mourelle and Ms Angelica Miranda for their technical support and advice in relation to mouse care and management and Dr Daniela Ureta for her assistance in flow cytometric analysis.

Competing Interests: None declared

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Legends to Figures

Figure 1. Inguinal lymph nodes (a) and spleen (b) total cell number and T cell populations detected at different times (24 hs and 196 hs) after skin exposure to an shUVd and to rlUVd, and in the non-irradiated control group. The figures show the percentage of CD_3^+, $CD_3^+CD_4^+$, $CD_3^+CD_8^+$ and $CD_3^+TCR\gamma\delta^+$ cells. * p<0,05; ** p<0,01, *** p<0,001. n = 5. The results are expressed as mean ± SD.

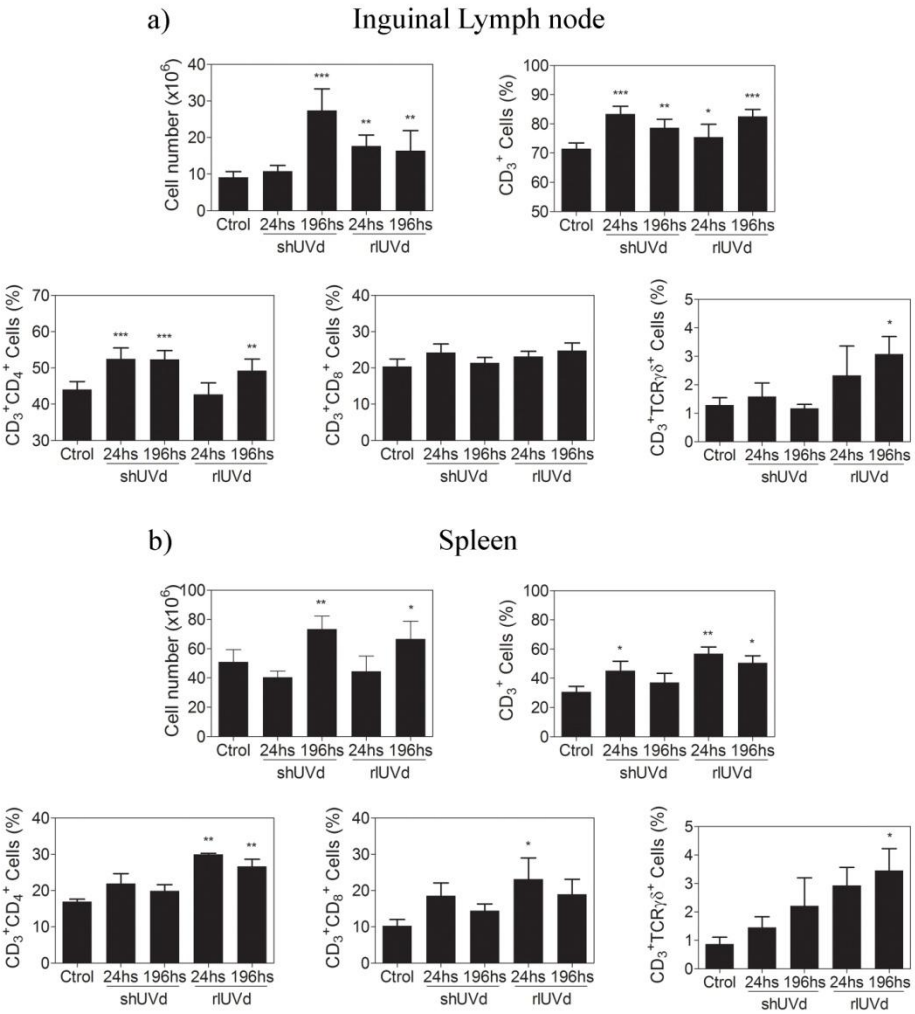
Figure 2. T cell function evaluated *in vitro* by unspecific stimulation and *in vivo* by contact hypersensitivity (CHS) reaction. Concanavalin A stimulation of inguinal lymph nodes (a) and spleen (b) cells after skin exposure to an shUVd and to rlUVd, and in non-irradiated control group. The values are expressed as proliferation index (PI). PI is defined as the ratio between stimulated and basal cell culture proliferation ($PI = OD_{540nm(X\ \mu g/ml\ ConA)} / OD_{540nm\ basal\ proliferation}$). IFN-γ, 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) CHS reaction performed 24 hours 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 hours after elicitation and the baseline ear thickness. * p<0,05; ** p<0,01, *** p<0,001. n = 6. The results are expressed as mean ± SD.

Figure 3. Anti-tetanus toxoid (TT) specific antibodies production. Mice were subcutaneously immunized with TT 24 hs or 196 hs after skin exposure to an shUVd and rlUVd, and in non-irradiated control group.. Three weeks later, specific IgM, IgG, IgA and IgG sub-isotypes were measured in sera. The titer was determined as EC_{50}, when the titration curves were complete, or as a Cut-off, when the titration curves resulted incomplete. The Cut-off was obtained as the triple of the minimal signal (bottom $OD_{450\ nm}$ x 3). * p<0,05; ** p<0,01, *** p<0,001. n = 5. The results are expressed as mean ± SD.

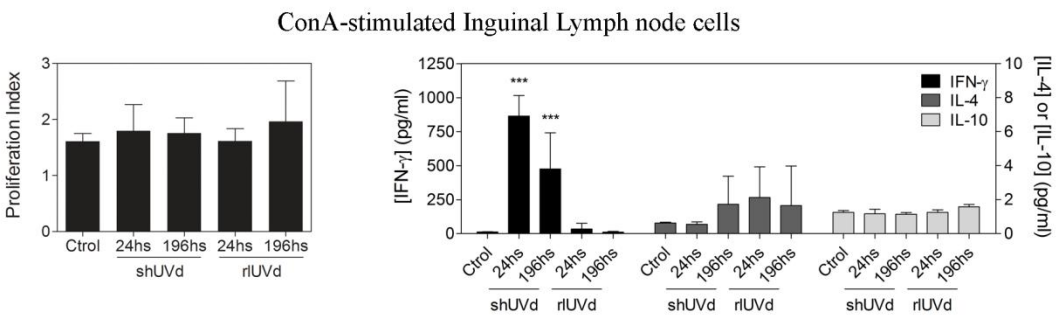
Figure 4. Daily evaluation of animal weight (a, b) and abscess size (c) during *S. aureus* infection. IL-6 levels were measured in sera previously to the inoculation, 5 hours and 6 days post-inoculation (d). * p<0,05; *** p<0,001. Ctrol: n = 7; shUVd: n = 5; rlUVd: n= 6. The results are expressed as mean ± SD.

Figure 5. Evaluation of local infection and inflammatory response in irradiated and control *S. aureus*-infected mice. Six days after *S. aureus* inoculation, the final abscess area and the bacterial count were determined (a). TNF-α, IL-6, IL1-β and CXCL-1 levels were measured in abscesses homogenates (b). Results are expressed as pg cytokine / mg protein. * p<0,05. Ctrol: n = 7; shUVd: n = 5; rlUVd: n= 6. The results are expressed as mean ± SD.

Figure 6. Evaluation of systemic infection and immune response in irradiated and control *S. 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 IFN-γ, IL-4 and IL-10 quantification in culture supernatant. c) Specific IgM and IgG titer in serum, six days post-*S. aureus* inoculation. * p<0,05. Ctrol: n = 7; shUVd: n = 5; rlUVd: n= 6. The results are expressed as mean ± SD.



a)



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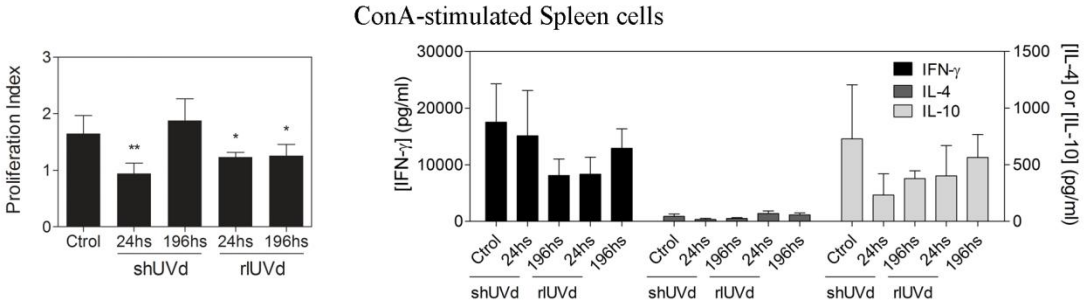

c)