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LETTER TO THE EDITOR

Lipoteichoic acid challenge induces higher inflammatory responses than lipopolysaccharide in UV-irradiated keratinocytes

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To the Editor,

Human skin is colonized by a diversity of bacteria, gram-positive mostly. Keratinocytes – the main cell population of the epidermis – not only act as a physical barrier but also participate in the defense against pathogen by activating the innate immunity to prevent pathogen invasion or survival, whereas they actively inhibit immune responses against resident microbiota. However, if the epithelium is disrupted, commensal bacteria are able to reach anatomical places where these balanced mechanisms are not present. Consequently, innate immunity mechanisms are activated (1–4). Keratinocytes express different innate immune receptors, such as toll-like receptors (TLRs), which play a crucial role in the induction of antimicrobial responses. TLRs recognize a wide variety of microbial compounds from gram-positive and gram-negative bacteria, such as lipoteichoic acid (LTA, present in gram-positive bacteria cell walls), recognized by TLR-2, and lipopolysaccharide (LPS, a major component of the outer membrane of the gram-negative bacteria), recognized by TLR-4. As a consequence of receptor–ligand interaction, keratinocytes produce different cytokines, chemokines, growth factors and antimicrobial peptides to control potential infections (5–9).

At the same time, skin is constantly exposed to harmful physical, chemical and biological agents. Among them, ultraviolet radiation (UVR) – the main skin carcinogen – impacts keratinocytes directly and stimulates them to produce pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α and IL-8, arachidonic acid-derived compounds and growth factors, among others, in response to this noxious stimuli (10, 11). UVR is also involved in cutaneous production of vitamin D_3. During sunlight exposure, cutaneous 7-dehydrocholesterol present in keratinocytes' plasma membrane is photochemically converted to pre-vitamin D_3. Epidermal keratinocytes express the complete enzymatic pathway needed to produce the active metabolite, $1,25(OH)_2VitD_3$. This vitamin acts as a transcription factor, regulating the synthesis of genes involved in the antimicrobial response besides bone metabolism effects (12). One of the pathways positively modulated by $VitD_3$ is the TLR-2 one, increasing the expression of this receptor in keratinocytes (13).

The way UVB influences the normal response of the epidermis to commensal and potential pathogenic bacteria is not well understood. The aim of the present work was to evaluate the antimicrobial response of UV-irradiated keratinocytes exposed either to LTA or LPS (representing gram-positive and gram-negative components, respectively) by means of pro-inflammatory cytokine production – IL-6 and TNF-α. Moreover, as LTA exerts its effects on keratinocytes via TLR-2 pathway, we decided to evaluate the role of the vitamin D pathway in our model.

A human keratinocyte cell line, HaCaT, was kindly provided by Dr. N. E. Fusenig (German Cancer Research

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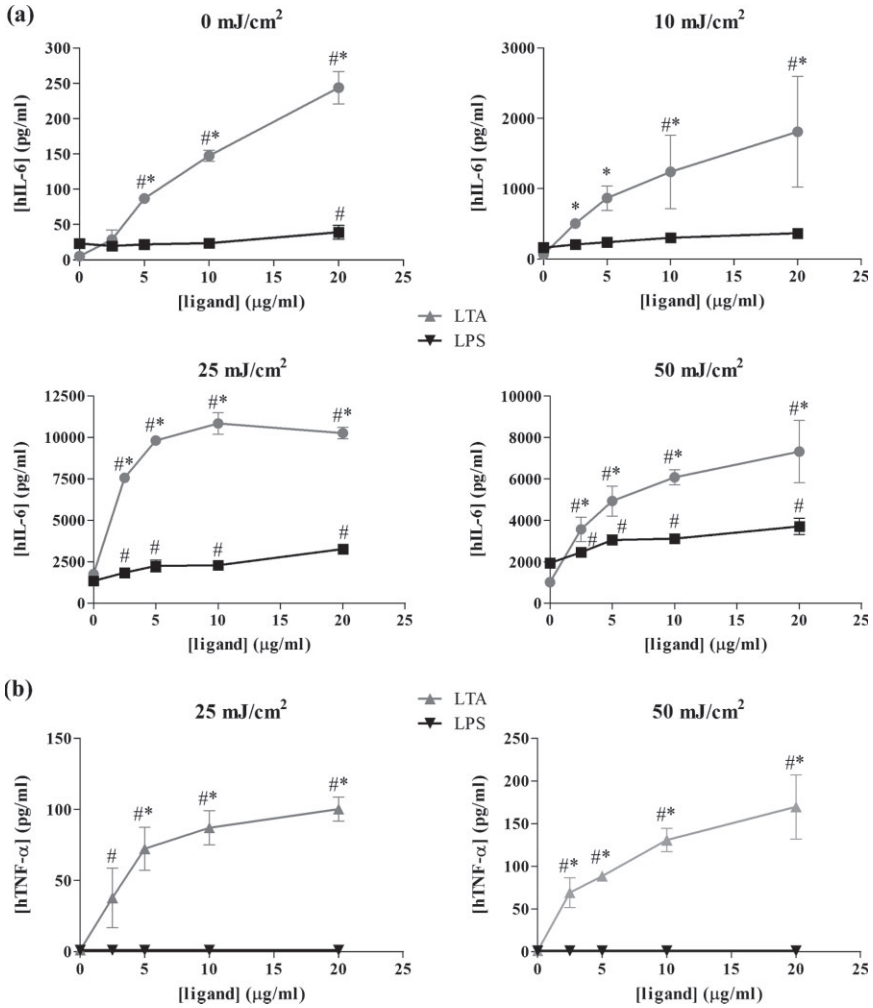

Fig. 1. Cytokine production in lipoteichoic acid (LTA) or lipopolysaccharide (LPS)-treated HaCaT cells. Interleukin (IL)-6 (a) and tumour necrosis factor (TNF)-α (b) were evaluated in culture supernatant from unirradiated (0 mJ/cm^2) or ultraviolet (UV)-irradiated (10, 25 and 50 mJ/cm^2) HaCaT cells. Cells were stimulated with LTA (gray lines) or LPS (black lines) at different concentrations. Differences against control (0 μg/ml) were analyzed by ANOVA (#$P < 0.05$) for each treatment (LTA or LPS). Differences between LTA and LPS were analyzed at each concentration with a Student's *t*-test (*$P < 0.05$).

Center, Heidelberg, Germany). HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand island, NY, USA) supplemented with 10% fetal bovine serum (Natocor, Córdoba, Argentina) and antibiotic solution (Penicillin–Streptomycin–Glutamine; Gibco-BRL, Grand island, NY, USA). Cells were grown in plastic flasks at 37°C in a humidified atmosphere containing 5% CO_2, with medium changes every 2–3 days. Subconfluent HaCaT cells were harvested with a pre-incubation with ethylenediaminetetraacetic acid (EDTA) 0.05% at 37°C for 15 min, followed by an incubation with EDTA–trypsin 0.05% for 2 min at 37°C (Sigma Chemical Co., St. Louis, MO, USA), and 5×10^4 cells per well were placed thereafter in 24-well plastic plates to perform all assays.

Previously to the irradiation procedure, sub-confluent HaCaT cells were washed with phosphate buffered saline (PBS), which is free of any photoactive compounds. Cells were exposed, with a minimum volume of PBS, to different UVR doses, using an 8W UVM-28 Mid-Range Wave (302 nm) lamp (emission spectrum 280–370 nm) from Ultraviolet Products (UVP, Upland, CA, USA). The lamp was calibrated with an UVX radiometer (UVP); its power was determined as 1.1 mW/cm^2. Therefore, cells were exposed 9 s, 23 s and 45 s to generate a dose of 10, 25 and 50 mJ/cm^2 of UVB, respectively. Several plates were not exposed to UVB radiation but were handled in the same fashion as the irradiated cells.

After irradiation or mock irradiation, the PBS was immediately replaced with DMEM containing different concentrations of LTA from *Bacillus subtilis* (less than 0.25 EU/ml assays by Limulus amebocyte lysate-LAL-test) or LPS from *Escherichia coli* (both from Sigma, St. Louis, MO, USA): 20, 10, 5 and 2.5 μg/ml and the corresponding controls with culture medium. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h, and then the supernatants were collected. These were stored at −70°C until they were used to evaluate different inflammatory mediators released to the medium by keratinocytes.

HaCaT cells were cultured in the same fashion as previously described. Subconfluent cells' medium was replaced

with DMEM containing different concentrations of 25(OH)Vitamin D_3: 1×10^{-7}, 1×10^{-8} and 1×10^{-9} M, in the presence or absence of LTA (10 μg/ml) as previously determined. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h, and then, the supernatants were collected and stored at −70°C.

Human TNF-α and IL-6 from HaCaT cells' supernatant were quantified using non-competitive ELISA assays (BD Biosciences, San José, CA, USA), according to the manufacturer's instructions. Values were expressed as picograms per milliliter.

Graphical and statistical analyses were performed with GRAPHPAD PRISM and GRAPHPAD INSTAT (GraphPad Software, Inc., La Jolla, CA, USA), respectively. Analysis of variance (ANOVA) followed by a Dunnet post-test was performed to make multiple comparisons against a control value and a Student's *t*-test to compare means of two groups. Results are expressed as mean ± SD; asterisks indicate significant at $^*P < 0.05$.

Keratinocyte exposure to UVR had a direct impact on IL-6 production. UVR increased HaCaT IL-6 production in a dose-dependent fashion (23, 160, 1341 and 1936 pg/ml for 0, 10, 25 and 50 mJ/cm^2, respectively). However, TNF-α was not detected at any UVR condition evaluated. LTA stimulation of HaCaT cells during 24 h increased IL-6 production in the same dose-dependent fashion as UVR (Fig. 1a). However, LTA had no effect on TNF-α production in unirradiated or low-dose (10 mJ/cm^2) irradiated HaCaT cells (not shown). Interestingly, we observed a synergistic effect between LTA and UVR for both IL-6 and TNF-α, which was more evident at 25 and 50 mJ/cm^2 (Fig. 1a and b, respectively). A similar response was obtained when HaCaT cells were treated with LPS, but only for IL-6 production (Fig. 1a). However, the magnitude of the response was lower than the effect obtained with LTA.

With respect to the correlation between the effect of the irradiation and the cell treatment with $VitD_3$, it was observed that 25(OH)$VitD_3$ produced similar effects to UVR on IL-6 production during LTA stimulation in unirradiated HaCaT cells (Fig. 2a–c).

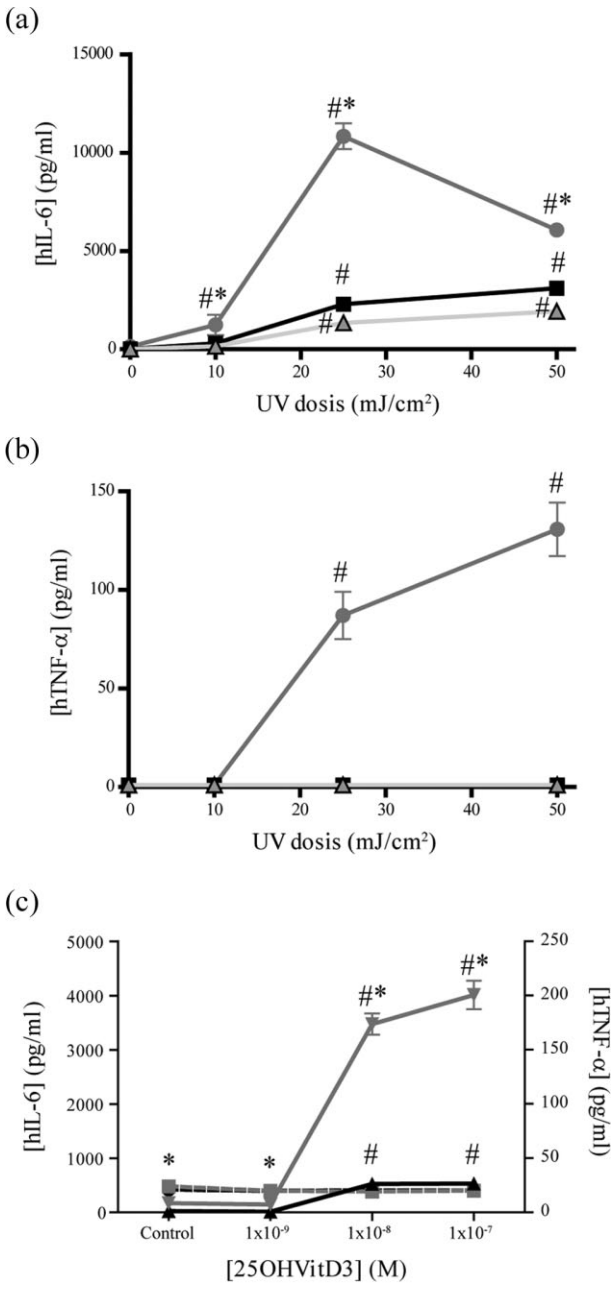

Fig. 2. Cytokine production in ultraviolet (UV) or vitamin D-treated HaCaT cells. (a) Interleukin (IL)-6 production and (b) tumour necrosis factor (TNF)-α production in lipoteichoic acid (LTA)-treated (gray line), lipopolysaccharide (LPS)-treated (black line) or control (light-gray line) HaCaT cells exposed to different UV doses. (c) IL-6 (solid line) and TNF-α (dotted line) production in LTA-treated (gray line) and control (black line) HaCaT cells stimulated with different doses of 25-(OH)-Vitamin D_3. Differences against control were analyzed by ANOVA ($\#P < 0.05$) for each treatment (LTA, LPS or control). Differences between LTA and LPS were analyzed at each concentration with a Student's *t*-test ($^*P < 0.05$).

DISCUSSION

Skin is the first line of defense against microorganisms. Although human skin is constantly exposed to a diversity of potential pathogenic microorganisms, it gets only rarely infected. The normal skin microflora includes mostly gram-positive bacteria, which are tolerated on the epidermal surface but induce inflammation when they reach the dermis. UVB is considered one of the most harmful stimuli

of the environment that causes direct damage on the epidermis leading to an inflammatory state. The impact of UVR on the normal antimicrobial response of keratinocytes has not been fully investigated.

In the present work, we aimed to evaluate how UVR exposure could affect keratinocytes' antimicrobial inflammatory response to gram-positive and gram-negative bacteria components, such as LTA and LPS. We observed that UVR increased keratinocytes' responsiveness to microbial external components. Interestingly, this response was more intense to LTA than to LPS, in contrast to the response commonly observed in other cell types like macrophages, where the immune response is bias towards gram-negative bacteria (14, 15). This increase in the response to microbial components, especially to LTA, was only partially observed in $VitD_3$-treated cells showing that other molecules might also be involved in this response. The preferential recognition of gram-positive bacteria can be considered as a specialization of the epithelium to its microflora. If this epithelium suffers an injury (like exposure to harmful radiation), it has to modify its response to microorganisms in order to avoid their entry into sites where they might produce infections. In this context, the increment in the inflammatory response observed in UVR-irradiated LTA-challenged keratinocytes is a novel evidence to understand the complex balance between the skin, its microflora and the environment.

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