Published in final edited form as:
Exp Dermatol. 2020 January ; 29(1): 102–106. doi:10.1111/exd.14044.

Ex vivo culture of mouse skin activates an interleukin 1 alpha-dependent inflammatory response

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Abstract

Ex vivo culture of mouse and human skin causes an inflammatory response characterized by production of multiple cytokines. We used ex vivo culture of mouse tail skin specimens to investigate mechanisms of this skin culture-induced inflammatory response. Multiplex assays revealed production of interleukin 1 alpha (IL-1α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), chemokine C-X-C motif ligand 1 (CXCL1), granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) during skin culture, and quantitative PCR revealed transcripts for these proteins were also increased. Ex vivo cultures of skin from myeloid differentiation primary response 88 deficient mice (*Myd88*$^{-/-}$) demonstrated significantly reduced expression of transcripts for the aforementioned cytokines. The same result was observed with skin from interleukin 1 receptor type 1 deficient mice (*Il1r1*$^{-/-}$). These data

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AUTHOR CONTRIBUTIONS

HZ, RMS, BG, MHK, DFS and MJT designed the research studies. SP, MHK, DFS and MJT contributed essential reagents and tools. HZ, RMS, LJS, MCB, PD, JA, WW III, AD and MJT performed the experiments. HZ, RMS and MJT performed data collection. MJT performed formal data analyses. DFS, MHK and MJT supervised these studies. MJT was responsible for writing the original draft, and HZ, RMS, LJS, MCB, PD, JA, WW III, AD, BG, SP, MHK, DFS and MJT contributed to reviewing, editing and formulating the final manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

suggested the IL-1R1/MyD88 axis is required for the skin culture-induced inflammatory response and led us to investigate the role of IL-1α and IL-1β (the ligands for IL-1R1) in this process. Addition of IL-1α neutralizing antibody to skin cultures significantly reduced expression of *Cxcl1*, *Il6* and *Csf3*. IL-1β neutralization did not reduce levels of these transcripts. These studies suggest that IL-1α promotes the skin the culture-induced inflammatory response.

Keywords

cytokines; explant; IL-1α; keratinocytes; mice

1 | BACKGROUND

The clinical phenomena of Koebnerization and pathergy in inflammatory skin diseases and the inflammatory phase of wound healing highlight the ability of skin injury to induce skin inflammation.[1] The mechanisms that regulate skin injury-induced inflammation are still being defined. Understanding these mechanisms and how dysregulation of these mechanisms can contribute to dermatologic diseases could lead to improved therapeutics for a variety of conditions. Interestingly, injury caused during skin biopsy collection (ie when this skin is incised with a biopsy tool) leads to a sterile inflammatory response in ex vivo cultured skin specimens; here, we will refer to this as the skin culture-induced inflammatory response.[2–5] This phenomenon suggests ex vivo culture of skin may be a useful approach to dissect the molecular and cellular mechanism by which skin injury promotes an inflammatory response and is the basis for the current studies. Pharmacologic studies by Roupe et al suggested the aforementioned inflammatory response is dependent on epidermal growth factor receptor (EGFR) activation; however, this same study indicated that the inflammatory response in mouse skin did not require EGFR activation.[5] Thus, the mechanism by which skin injury promotes inflammation in ex vivo cultured mouse skin remains to be determined.

2 | QUESTIONS ADDRESSED

1. What is the profile of cytokine transcript and protein production during the first 24 hours of ex vivo culture of mouse tail skin?
2. How does IL-1 signalling contribute to the skin culture-induced inflammatory response?
3. Which IL-1 (ie IL-1α or IL-1β) is required for the skin culture-induced inflammatory response?

3 | EXPERIMENTAL PROCEDURES

These investigations used adult male wild type (WT), *Myd88*$^{-/-}$, *Il1r1*$^{-/-}$, *Tlr2*$^{-/-}$ *Tlr3*$^{-/-}$ or *Tlr4*$^{-/-}$ mice (C57BL/6; The Jackson Laboratory) maintained under specific pathogen-free conditions and were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. After euthanasia with carbon dioxide, mice were placed in Wescodyne (1:40; water) for 5 minutes, washed in sterile water, incubated for 3 minutes in

70% ethanol and washed twice more in water. Tail skin was then removed in one piece followed by collection of 6 mm punch biopsy specimens under sterile conditions. Specimens were snap-frozen in liquid nitrogen immediately after collection or after culture for the indicated time points at 37°C and 5% CO_2 in EpiLife media (Thermo Fisher Scientific). Skin was then processed for RNA isolation, cDNA synthesis and TaqMan™ qPCR (Thermo Fisher Scientific), and culture supernatants were collected for multiplex assays. Mouse ubiquitin protein c (*ubc*) mRNA was the internal control for qPCR. The Mouse Cytokine Array/Chemokine Array 44-Plex multiplex platform (Eve Technologies) was used to measure cytokine production in skin culture supernatants.

4 | RESULTS

During ex vivo culture of mouse skin, CXCL1, IL-6, IL-1α, IL-1β, GMCSF (CSF2) and G-CSF (CSF3) concentrations increased significantly (Figure 1A). To determine whether the transcripts encoding these proteins changed similarly, qPCR was performed with cDNA from WT skin snap-frozen immediately after biopsy or cultured for 4 or 24 hours. Quantities of *Il6* and *Csf3* significantly increased at the 4 hours culture time point, and *Cxcl1*, *Il6*, *Il1a* and *Csf2* levels were significantly increased at 24 hours (Figure 1B). Although *Il1b* transcripts did not increase significantly during culture, there was a trend towards increased expression similar in magnitude to the small but significant increase in IL-1β protein at 24 hours, suggesting increased IL-1β could be secondary to increased *Il1b*. Alternatively, inflammasome activation could account for the increase in IL-1β. In summary, these data demonstrate the inflammatory response activated by ex vivo skin culture is characterized by increased cytokine transcript and protein production.

Next, the mechanisms that promote the skin culture-induced inflammatory response were investigated. Cellular injury in many organ systems, including the skin, can liberate alarmin molecules (eg IL-1α, IL-33, high mobility group box 1 protein) that promote inflammation via activation of Toll-like or IL-1 family cytokine receptors.[6] As most of these receptors require the adaptor protein MyD88, ex vivo culture experiments were performed with skin from MyD88 deficient (*Myd88*$^{-/-}$) mice.[7] Skin from *Myd88*$^{-/-}$ mice demonstrated significantly reduced levels of skin culture-induced inflammatory response cytokine transcripts (Figure 2A). To determine whether IL-1 signalling is involved in activation of this response, ex vivo culture studies were performed with skin from IL-1R1 deficient mice (*Il1r1*$^{-/-}$) and demonstrated significantly reduced levels of cytokine transcripts compared with WT skin (Figure 2B), indicating the IL-1R1/MyD88 axis is required for the skin culture-induced inflammatory response. Conversely, toll-like receptors TLR2, TLR4 and TLR9 (MyD88 dependent) were not required for the skin culture-induced inflammatory response (Figures S1 and S2). The MyD88-independent toll-like receptor, TLR3, was also not required for this response, with the possible exception of *Cxcl1,* which was reduced at the 24 hours time point in one of two experiments with skin from *Tlr3*$^{-/-}$ mice (Figure S3).

The IL-1R1 protein is the ligand-specific subunit of the pro-inflammatory IL-1 receptor.[8] Based on the *Il1r1*$^{-/-}$ mouse studies, IL-1α and IL-1β were initially considered as potential activators of the skin culture-induced inflammatory response. In the skin, IL-1α is constitutively produced and stored in keratinocytes and can be released from damaged cells

to promote inflammation.[9] Unlike IL-1α, IL-1β is not abundant in "healthy skin," requires several steps for production (ie transcription, inflammasome activation, caspase 1-mediated cleavage and secretion) and is abundantly produced by neutrophils, monocytes and macrophages, rather than keratinocytes.[8,9] Based on these factors and the rapid induction of cytokine transcripts seen during skin culture, preformed IL-1α was hypothesized to be the primary stimulus for activation of the skin culture-induced inflammatory response. To test this, WT mouse skin was cultured with validated (Figure S4) neutralizing monoclonal antibodies to mouse IL-1α or IL-1β or isotype control followed by qPCR for skin culture-induced inflammatory response transcripts. In ex vivo skin culture, IL-1α neutralizing antibody significantly reduced *Cxcl1*, *Il6* and *Csf3* levels, while reductions in *Csf2* and *Il1b* were observed but not significant (Figure 2C). IL-1β neutralizing antibody did not reduce *Cxcl1*, *Il6*, and *Csf3* levels and even increased *Il1a* and *Il1b*, possibly through a loss of a negative feedback effect of IL-1β in this system.

Immunohistochemical studies demonstrated diffuse staining for IL-1 α in the epidermis of mouse tail skin prior to culture (Figure S5). In addition, the IL-1R1/MyD88 dependent transcripts, *Cxcl1*, *Il6* and *Csf3*, but not *Il1a*, were induced within 4 hours of culture (Figures S6 and S7). Increased levels of *Il1a* and *Csf2* (also IL-1R1/MyD88 dependent) were not observed until the 24 hours time point. These data suggest preformed, rather than newly synthesized, IL-1α induces *Cxcl1*, *Il6, Csf3*, and possibly, *Il1b* expression, while *Il1a* and *Csf2* induction requires an additional factor (eg cytokine, stressor) generated during skin culture.

5 | CONCLUSIONS

These studies provide a broader understanding of the inflammatory response induced during ex vivo culture of mouse skin and implicate IL-1α-mediated activation of IL-1R1/MyD88 signalling as a mechanism for activation of this response. Although it is not certain whether commensal (or even pathogenic) microbes can alter the skin culture-induced response, studies by Naik et al demonstrated *Il1a* expression in keratinocytes from germ-free mice was similar to that seen in keratinocytes from specific pathogen-free mice.[10] This coupled with the toll-like receptor deficiency/inhibition experiments described here, suggest host microbes may not impact the skin culture-induced inflammatory response, though further studies are needed to address this issue. Future studies are also needed to address the contribution of IL-1α to human skin culture-induced inflammation.

This work has several implications. First, it highlights that skin resident cells are sufficient to promote at least some key aspects of inflammation in cultured skin (ie in the absence of infiltrating inflammatory cells) and provides an experimental platform to identify the cellular sources and targets of skin culture-induced inflammatory response cytokines. In addition, attenuation of the skin culture-induced inflammatory response using IL-1α blockade could help "preserve" the in vivo skin phenotype, making cultured skin a better model for studying cutaneous biology and pathology. Finally, the skin culture-induced inflammatory response, defined here, may represent part of the inflammatory phase of wound healing seen in vivo. If this is the case, ex vivo culture of skin could be used to study the contributions of

keratinocytes, fibroblasts and other resident skin cells to the inflammatory phase of wound healing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by the Dermatology Foundation [Physician-Scientist CDA] to MJT; The Department of Veterans Affairs [VA CDA2; IK2 CX001019] to MJT; The Ralph W. and Grace M. Showalter Research Trust to MJT; a generous contribution from Dr Stephen Shideler in support of the IUSM Department of Dermatology/MJT; and National Institutes of Health grants to SP [R21 HL139934], MHK [R01 AI095282], DFS [R01 AG048946 and ES020866] and RMS [T32 AR062495]. We would also like to thank Dr Andrzej Slominski for his help in editing the manuscript.

Funding information

National Institute on Aging, Grant/Award Number: R01 AG048946; U.S. Department of Veterans Affairs, Grant/Award Number: IK2 CX001019; National Heart, Lung, and Blood Institute, Grant/Award Number: R21 HL139934; National Institute of Allergy and Infectious Diseases, Grant/Award Number: R01 AI095282; Ralph W. and Grace M. Showalter Research Trust Fund; National Institute of Arthritis and Musculoskeletal and Skin Diseases, Grant/Award Number: T32 AR062495; Dermatology Foundation, Grant/Award Number: CDA; National Institute of Environmental Health Sciences, Grant/Award Number: R01 ES020866

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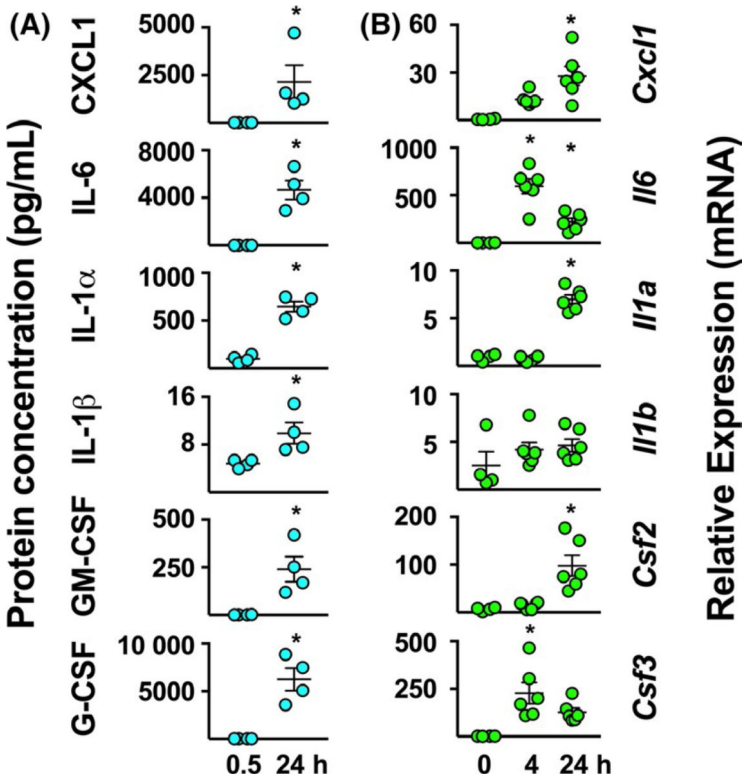

FIGURE 1.

Characterization of the skin culture-induced inflammatory response. A, Cytokine and chemokine protein concentrations in supernatants from ex vivo skin cultures were quantified with a Mouse Cytokine Array/Chemokine Array 44-Plex (Eve Technologies). Skin punch biopsies (6 mm) from WT mice were cultured for 0.5 or 24 h (n = 4) at 37°C and 5% CO_2 in EpiLife media containing 20 μmol/L $CaCl_2$. B, Indicated transcript levels were determined by qPCR (TaqMan™) using mRNA from mouse tail skin cultured for 4 or 24 h (n = 6) or skin that was collected for RNA prior to culture (0 h; n = 4). Statistical significance was determined using a two-tailed *t* test (A) or one-way ANOVA with Dunnett's multiple comparison test (B). Data are expressed as SEM. **P* < .05

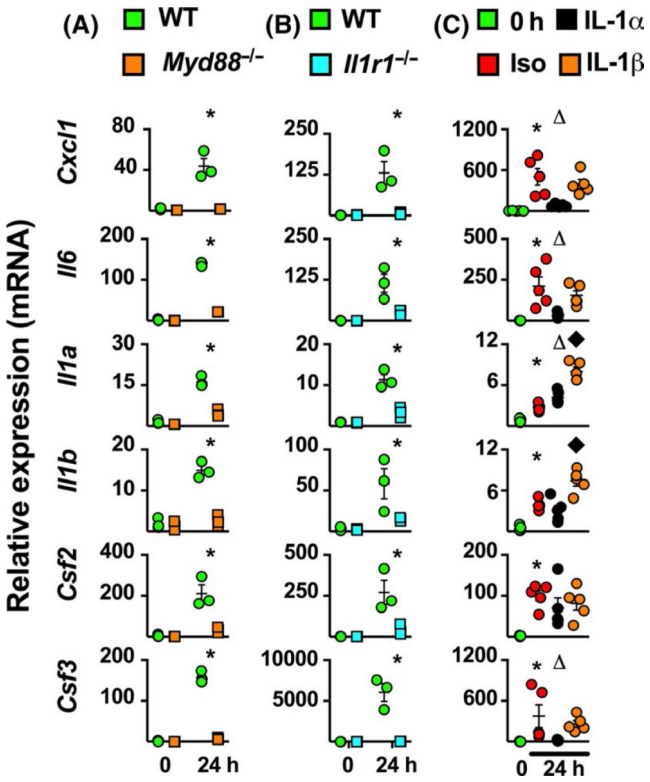

FIGURE 2.

Role of IL-1α in skin culture-induced inflammatory response. Skin from (A) WT and *Myd88*$^{-/-}$ or (B) WT and *Il1r1*$^{-/-}$ mice was cultured for 0 or 24 h (n = 3/group). C, WT skin was processed immediately for RNA (0 h) or preincubated at 4°C for 4 h in media containing 5 μg/mL hamster IgG (Iso; 400969; BioLegend) control or anti-mouse IL-1α (IL-1α; MAB4001; R&D systems) or IL-1β (IL-1β; MAB4012; R&D systems) neutralizing antibodies followed by culture at 37°C for 24 h (n = 5/group). Two-way ANOVA with Sidak's multiple comparisons (A, B) or one-way ANOVA with Dunnett's multiple comparisons (C) tests was used. Data are expressed as SEM $P < .05$ for (A) *WT vs *Myd88*$^{-/-}$, 24 h; (B) *WT vs *Il1r1*$^{-/-}$, 24 h; (C) *0 h vs Iso, $^{\Delta}$Iso vs IL-1α and ♦Iso vs IL-1β