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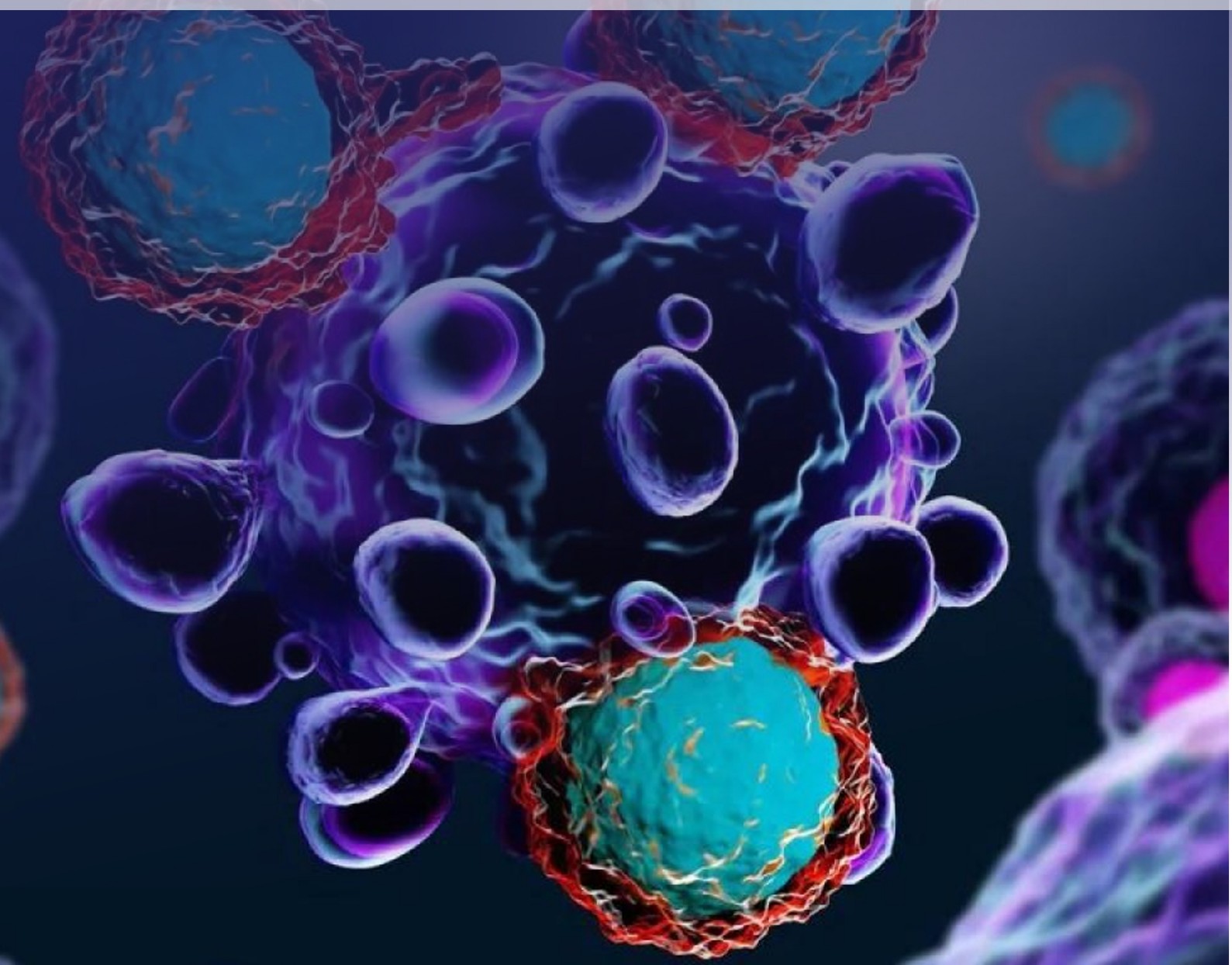

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EDITORIAL

CAR-T therapy is a breakthrough for intractable autoimmune disease?

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The dramatic effects of checkpoint inhibitor therapy[1,2] and chimeric antigen receptor T (CAR-T) cell therapy[3,4,5] have attracted a great deal of global attention and have recapitulated the history of cancer treatment so far. The recent approval of two CAR-T therapies by US Food and Drug Administration marked a very significant development in cell-based cancer immunotherapy. This milestone was demonstrated by the effectiveness of eradicating hematologic cancers using CD19-specific CARs.

The success spurred development of immune cell therapies for other cancers, especially solid tumors. With regard to CAR-T therapy for solid tumors, although the clinical effects are limited, some unexpected serious adverse events have been reported and there are still many problems. The generation of novel CAR constructs for these cancer types represents a major challenge in bringing the technology 'from-bench-to-bedside'. In the review of Santos and Bernal[6], they outlined some new technologies to equip CAR-T cells to enhance efficiency while decreasing toxicity of CAR-T therapies in solid tumors. Development research on safer and more effective CAR-T therapy for solid tumors is in progress and will improve the outcome of treatment for patients with refractory leukemia and cancer in the future.

Furthermore, it was expected that this CAR-T method would be applied to intractable autoimmune diseases. In the recent article of Kansal R *et al*[7], CD19-targeted CAR-T therapy is reported to be highly promising for intractable systemic lupus erythematosus. They used murine lupus models such as in the (NZB × NZW) F1 and MRL lpr/lpr mouse. New Zealand (NZ) mouse and MRL lpr/lpr mouse are well known to be a B-lupus model and a T-lupus model, respectively. The clinical trials of anti-CD20 antibody for lupus failed because of the transient and incomplete B cell depletion. They reported that CD8+ T cells expressing CD19-targeted CARs persistently depleted CD19+ B cells, eliminated autoantibody production, reversed disease manifestations in target organs, and extended life spans in murine lupus models. For human application, there will be many issues to be solved, but at least it will lead to the elucidation of the pathogenesis of intractable autoimmune diseases.

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EDITORIAL

Type I IFNs and *M. bovis* BCG vaccine: The sword to target germinal centers and unlock humoral immunity in leprosy

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ABSTRACT

Leprosy is still a serious human health problem in developing countries. Environmental and genetic factors are playing a key role in the chronic course of the disease, resistance versus susceptibility. Multidrug treatment is not effective for all infected individuals. "Cured" individuals mostly show relapses of neurological disorders as the same as not cured can present physical and deformed constraints. The unsolved matter in leprosy is that the clinical spectrum depends on the host immune response.and thus, the outcome of the immune response. In the present review we intend to describe some aspects of the immunotherapy, based on type I IFNs and *M. bovis* BCG vaccine like a sword strategy to target germinal centers, either for its generation or for its enhancement and thus, throughout key signals delivered by follicular CD4+ T cells and controlled by follicular regulatory CD4+ T cells, B cell differentiation into plasmacytoid cells being highly promotes the induction of protective high affinity neutralizing antibodies to unlock humoral immunity towards *M. leprae* infected individuals.

Keywords: *type I IFNs; M. bovis BCG; germinal centers; leprosy; humoral immunity*

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Introduction

Hanses's infection disease (human leprosy) caused by Mycobacerium leprae is an ancient world infectious disease[1,2] that still constitutes a serious threat to human beings in developing countries[1-3]. The multidrug treatments are not effective for all infected individuals. The genetic susceptibility to the disease as well as the environmental (poverty, geographic localization) plays a pivotal role in the development of the clinic manifestations and the outcome of the host immune response[4-7]. How and whether or no epigenetic factors influence the outcomes in a manner positive or negative, are issues that deserve further studies[8,9]. A major limitation that has dampened advances in the understanding of the immunopathogenesis of leprosy is that M. leprae could not be propagated in vitro. However, nowadays, cellular immunology of leprosy can be approximated in vitro throughout keratinocytes infection[10] as well as human airway epithelial cells[11]. Moreover, tick cell lines constitute a step forward to a promising venue not only for large scale production of M. leprae bacteria but for deep insight into the mechanism of pathogenicity[12].

M. leprae is an alcohol acid resistant intracellular bacilli pathogen[9,10] comprised of at least 33 immunodominant antigens (genome scan)[12], among them a 19 and 33Kda lipoproteins that interact through pattern pathogen receptors (PRRS), Toll-like receptors on innate cells (macrophages, dendritic cells)[13-15] to trigger an acute pro-inflammatory state accompanied with TNF-alpha and IL-12 to connect with adaptive immune response (CMI)[16-19]. In leprosy either in humans (*M. leprae*) or in mice (*M. lepraemurium*), the host-pathogen interaction leads to a spectrum of cellular immune and clinical phenotype[18-20]. As outlined in

Figure 1, in one extreme of the spectrum, tuberculoid (TT/) on strong Th1 and Th17 immune response that limits the pathogen and dissemination. In the middle, two intermediate phenotypes, Borderline tuberculoid (BT) and Borderline (BB), a mix of a Th1 and Th2 cellular immune response. Moreover, TLR1, TLR2 enhanced expression in resistant infected patients (Tuberculoid Leprosy) and down regulation in susceptible infected (TLL)[5,19]. What determines that each of these intermediae stages is polarized toward a predominant Th1 or Th2?. On the other end of the spectrum a lepromatous disease or borderline lepromatous leprosy with a Th2 type cellular immune response, and a regulatory T cellular component (CD4-FoxP3+) that can not limit the dissemination of the pathogen and is dominated by a state of anergy (**Figure 1**). Indeed, leprosy regulatory T or B cells leads to a immunosuppressive ambiance by IL-10, TGF-beta induction instead oof IL17 protective immune responses and avoid exacerbates helper follicular T cell as well as B cell proliferation[21-25]. The state of immunosuppression has been proposed to play a pivotal role in the immunopathogenesis and immunoregulation not only in human leprosy but also in autoinmune diseases[25]. The role of regulatory T cells is to control or regulate functions in different sites, however, in some infectious diseases such as leprosy it is intriguing that B and T regulatory T and B cells instead of immunoregulate promotes a immunosuppressive state for the establishment of the pathogen. Therefore, it is tempting to think that in eradicated regions, the treatments promoted a strong Th1 and Th17 immune protective response while in endemic regions, infected individuals have a dysregulation of the immune response, and the induction of a state of anergy that hampers elimination of the pathogen. How the Th1 cellular protective immune response can be augmented against disiduos complex pathogens like M. leprae?. How to resolve this immunological challenge and moreover, how to modulate it in order to obtain more beneficial protective responses than damage to the host. One hypothesis that raises from different studies is that vaccines adjuvants against infected individuals might be directed germinal centers because these sites are programmed for high affinity neutralizing protective antibodies induction either for bacterial or virus infections[26,27].

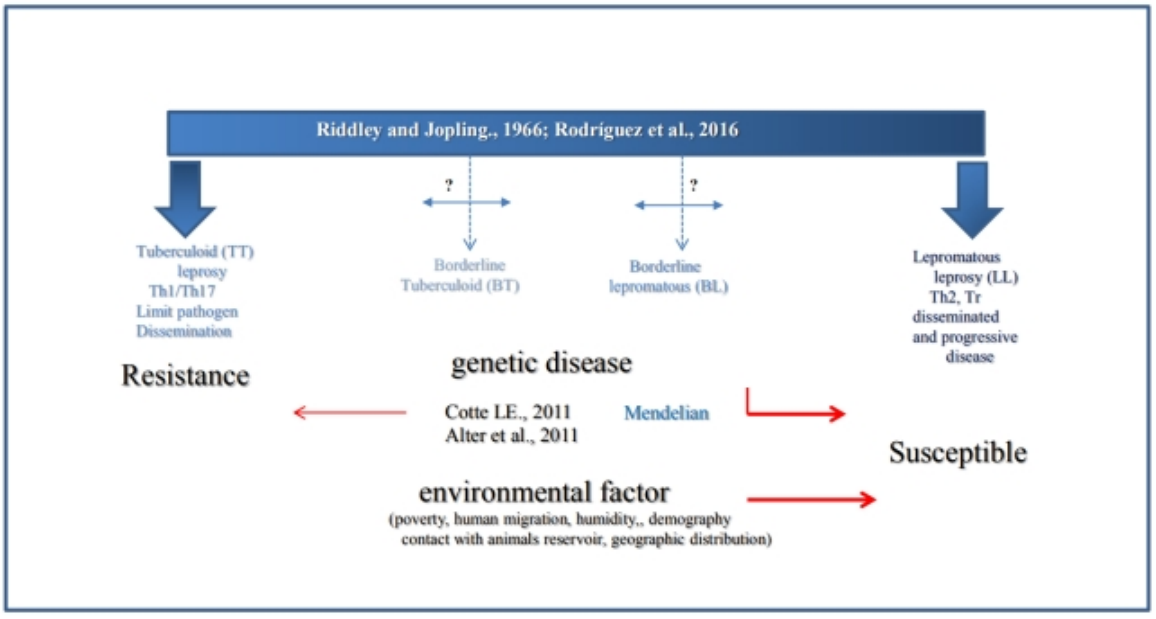

Figure 1. Outline of the clnical spectrum of the host immune response in leprosy**.**

Riddley and Jopling in 1966 defined Hanse's disease or leprosy,-caused by *Mycobacterium leprae*, -as a heterogenous infectious disease because it shows a wide spectrum of clinic manifestations. *M. leprae* is monomorphic, there are not divergent variability among strains, making more feasible to study and vaccine development. However, multidrug treatments are not effective for all infected individuals presumably because the environmental factors and genetic susceptibility to disease.

Germinal centers

Germinal centers (GCs), are niches inside secondary lymphoid organs (lymph node, spleen cells), that arise from the center of primary B follicles, programmed for the antigen diversification through B cell receptor (BCR) somatic hypermutation (SHM). This process can be simple for simple antigens (protein), few mutations are required, but in the case of a more complex pathogen (virus, bacteria), protective since high affinity antibodies require a much higher rate of mutations[26,27-29]. The program of the high antibody diversity generation is well ordered and not limited, includeing that follicular T cells provide key signals to B cells for plasmacytoid differentiation and memory B cells induction, clonal expansión, hypersomatic mutation, and class switch of class. The output of the GCs reactions depends on several factors, in between of the interaction of stromal follicular dendritic cells (FDCs), follicular B cells (FBC), follicular helper T cells (Tfh), follicular regulatory T cells (TFr), foamy macrophages in GCs[30-34]. The understanding of all GCs reactions that occur in secondary lymphoid organs (SLOs) or in tertiary lymphoid organs (TLOs) is pivotal for the development of vaccine[35-37]. GC reaction or generation of GC is an ordered process that involves different innate and adaptive cell populations, and all the reactions should be carefully regulated in order for rare antigens to be able to mount a high quality number of antibodies[35,36]. In the light of our data and from the literature, we think that this potential adjuvant vaccine (induction of GCs in secondary lymphoid tissues) is promising since the hallmark of vaccination and development of human vaccines are the induction of neutralizing protective antibody responses that depends strongly on CD4+ T cell population and germinal centers (programmed for antibody diversity generation, clonal expansión, hypersomatic mutation, switch of class), and thus, to have enhanced affinity and maturation of the humoral response which could primarily boost IgG subclass antibodies magnitude[38,39].

From the outside, the lifespan of GCs, can be modulated by adjuvants vaccines, by immunization protocols or strategies to long lastly Ag availability[37,38]. To approximate this first in *vivo* and then *in vitro*, we had used *M. lepraemurium* mice infection whose primarily and general clinic manifestation is skin lesión and peripheral nerve damage[16]. In murine leprosy at early times of infection, Th1 type cellular immune response limits the pathogen dissemination reflecting the tuberculoid phenotype. But weeks later, there is a dysregulation of either cellular and humoral immune response that last with a state of anergy giving rise to lepromatous phenotype[40-42].

Type I Interferons

In agreement with already data from the literature[43-47], we had found that the sole administration of type I IFNs exacerbate *M. lepraemurium* and/or M. tuberculosis infection. Firstly, skin lesión was not developed[48] while in the second a reduction of the bacterial load was observed[49]. Interestingly, we found in either infection model as well as from *in vitro* experiments (*M. tuberculosis*) that the treatment based on type I IFNS plus BCG vaccine promote strong Th1 cellular immune response and protection against *M. tuberculosis* infection[49], whereas in murine leprosy type I IFNs induced protection correlated with a high induction of iNOS induction[48], The mechanism of this induced protection in either model are studies that are currently addressed. As part of an approximantion of the anergy observed at the level of the celular and in the humoral immune response, we asked how adjuvants such as type I IFNs could have an influence on GCs processes in secondary or tertiary lymphoid organs?

Type I IFNs are induced at a very low steady level in the absence of bacterial or viral infection[43-45] (**Figure 2**). However, after infection (bacterial or viral) or immunization, type I IFNs signalization on innate cells (dendritic cells, DCs) thorough IFN receptor (IFNR) (**Figure 2**) induce the expression in mesenteric lymph node of CXCR5+ follicular T helper, cells to support plasmacytoid B cell differentiation into plasmablasts and plasma cells that secrete high affinity switched Abs as well as the generation of memory B cells,[22,23] leading thus to an enhanced cross protective immune response. CXCR5+ expression also enables B cells to be recruited to the lung and thus, initiate formation of functional GCs (**Figure 2**). Furthermore, type I IFNs after viral infection can induce CXCL-13 expression in lung fibroblast, conducting thus, to CXCR5+ dependent recruitment of B cells, leading to initiation of ectopic GCs formation[31,32]. This supports a role of type I IFNs as a natural adjuvant of SLO or TLO lung remodeling[31,32]. Is this a direct evidence that type I IFNs induce more efficient germinal centers in mice. It has been described that GCs enhancement and therefore humoral immune responses potency can be modulated throughout extended antigen availability favored by vaccine adjuvant and of the

immunization strategies[27]. The mechanism of this had been proposed to occur precisely as stated above through improving T follicular helper (Tfh) cell generation which will lead to enhanced affinity maturation and enhanced development of B ell memory. From our own findings, successive IFN-alpha boosting of *M. bovis* BCG vaccinated mice, increased GCs average in SLO that correlated with induced protection in intradermal infected mice (lack of skin lesion) (Guerrero *et al*., unpublished results). How type I IFNs natural action in infected *M. leprae* individuals is proposed to initiate; as outlined by[46], toll like receptors (TLR2-/1), Interferon 1 and 2 receptors (IFNR); intracellular nucleotide binding oligomerization domain containging -(NOD2)NOD-receptors, trigger a series of pathways which final commitment is the shut off or at least downregulation of pro-inflammatory response (macrophage microbicidal mechanisms, oxide nitric synthetase (iNOS, Il1-β, IL-6, TNF-α, IL-12, IFN-γ) against versus antiinflammatory respose (*M. leprae* or *M.tuberculosis* infection) represented by a suppressive state leaded by IL10; the stimulator of interferón genes (STING); interferón regulatory factor 3(IRF3, cGAS (cGMP-AMP syntahse); [2'-5'-oligoadenylate synthetase](OASL). But also there is a change in the energy supply through glucose uptake, pentose pathway that generates, nicotinamide adenine dinucleootide phosphate (NADPH), lipids metabolism (arachidonic acids, eicosanoids, leukotrienes), as well as an increase in mitocondrial activity that serve to the own pathogens purposes, a switch off in the metabolism to redirect carbon(s) source for nutrients survival inside lipid bodies) that it has been observed in the skin lesion of *M. leprae* infected patients[46,47] (**Figure 2**). Autophagy and a pro-inflammatory response are activated upon NOD2 recogniton of mycobacterial DNA, that initiate a leucine-rich-repeat kinase 2 (LRRK2)-dependent response followed by host's recruitment of ubiquitin chains, exemplified by E3-ubiquitin ligase parkin (PARK2) to target mycobacteria to autophagic degradation (**Figure 2**). The evasion mechanism used by is the early secreted system (ESTX-1) mediate pore formation in the autophagosomal membrane to equalize autophagosomal and cytosol content. This, eventually trigger type I IFNs response and autophagy and downregulation of IL1-β. Interestingly, as depicted in **Figure 2**, the sword represented with *M. bovis* BCG vaccine plus IFN-alpha propose that this strategy might be redirecting type I IFNs adjuvant properties toward an increase in the antigen availability favored at the same time through an increase in the CXCR5+CD4+T cell generation and further CXCL13+ expression in SLO or TLO follicular B and T cells, key players to unlock humoral immuner responses in GC.

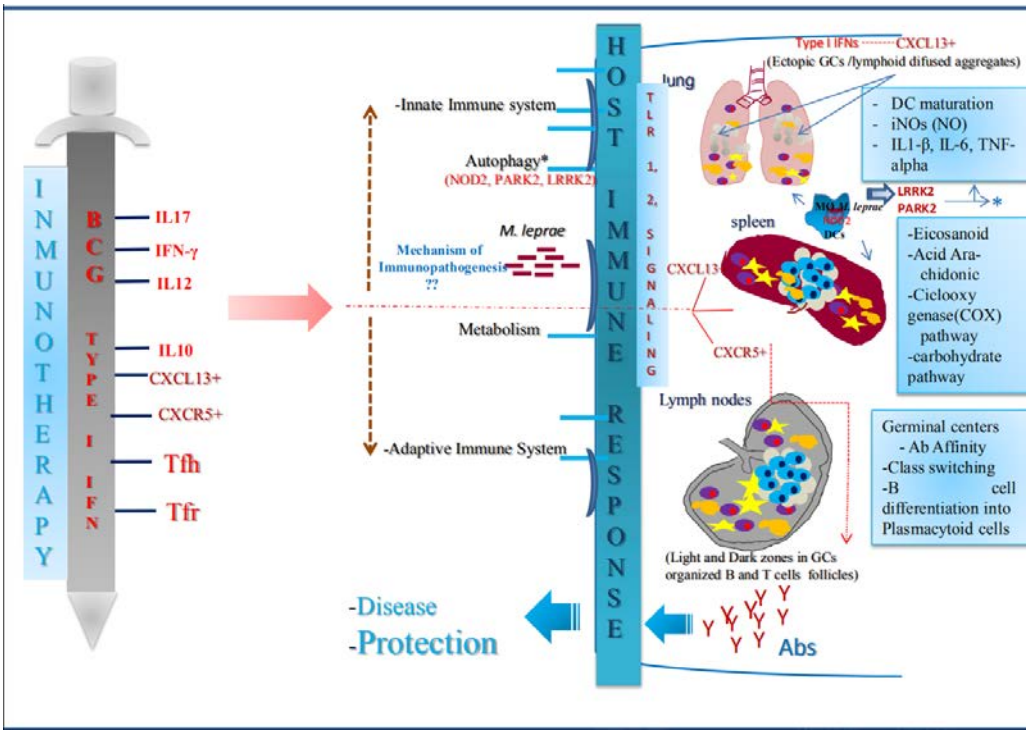

Figure 2. Scheme of type I IFNs and *M. bovis* BCG vaccine immunotherapy as a sword in the host immune response in leprosy**.**

In the *M. leprae* infection, the outcome of the immune response depends of the effectiveness in the generation of key innate and cellular host immune system at systemic and mucosal tissues. type I IFNs adjuvant action in synergy with *M. bovis* BCG vaccine faced host immune response, such as autophagy, metabolism upon *M. leprae* infection. Intracellular receptors such as nucleotide binding oligomerization domain containing 2 (NOD2) upon recognition of muramyl-dipeptides of *M. leprae* (first interaction with Toll-like receptors, TLR2/1) can initiate a leucine-rich-repeat-kinase 2(LRRK2)-dependent pro-inflammatory response as well as autophagy. While targeting of *M. leprae*/*M. tuberculosis* for autophagic degradation involves recruitment of the host ubiquitin system that depends on an E3-ubiquitin-ligase or parkin encoded by the gene PARK2, associated with leprosy susceptibility. Once the pathogen enter and switch the metabolism of glycolysis, lipids and cholesterol for its own purposes, it is highly possible that type I IFNs robust adjuvanticity plus *M. bovis* BCG redirect its action to unlock humoral immune responses throughtout enhancement of CXCL13+ (lung fibroblast) and CXCR5+ in lymph node to enhance follicular T helper cells (Tfh) cells that are primordial to deliver key signals for B cell differentiation in GCs to plasmacytoid cells for the production of high affinity neutralyzing antibodies.These processes are modulated by follicular regulatory T cells (Tfr). Moreover, it is tempting to think that the sword of the proposed immunotherapy pin-point the antigen availability potentially favored by the successive immunization strategy of IFN-alpha to *M. bovis* BCG vaccinated mice.

Conclusions

In the immunobiology of leprosy (*M. leprae*/*M. lepraemurium*), most of the studies have focused to search for therapeutic targets etiher from genome wide studies (GWS) or from microarray expression profiles which have pin point several genes of susceptibility or resistance as well as potential biomarkers of the different interrelated pathways such type I IFNs, autophagy and metabolism. But still remains unsolved others aspects in the biology of the disease. From our own findigns, successive IFN-alpha boosting of *M. bovis* BCG vaccinated mice, increased GCs average in SLO that correlated with induced protection (lack of skin lesión development) (Guerrero *et al*., unpublished results). As depicted in figure 2, the sword comprised of *M. bovis* BCG vaccine plus IFN-alpha propose that this strategy might be redirecting type I IFNs adjuvant properites toward an increase in the antigen availability favored at the same time through an increase in the CXCR5+CD4+T cell generation and further CXCL13+ expression in SLO or TLO follicular B and T cells, key players to unlock humoral immuner responses in GCs.

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ORIGINAL RESEARCH ARTICLE

Acute-phase effects of single-time topical or systemic corticosteroid application immediately after hot water-induced burn injury of various grades

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ABSTRACT

The aim of this study is to evaluate the effects of corticosteroid application on each grade of burn, and to clarify the underlying mechanisms of the effects, especially in its acute inflammatory phase. To generate three-graded burn models (epidermal burn, or EB; dermal burn, or DB; and subcutaneous burn, or SB), hot water was applied on the back skin of Hos:HR-1 mice. Strongest-class (or high-potent) corticosteroid ointment (DD group) or petrolatum (control group) was applied on the back immediately after the hot water application on mice. Prednisolone sodium succinate (PDN group), 1 mg/kg was orally applied immediately after the hot water application on mice. The mice were sacrificed 1–3 days after hot water application, and the lesional skin samples were provided for histological assessment to enumerate the number of infiltrating inflammatory cells. The mRNA expression levels of inflammatory cytokines (*IL-1β*, *TNFα*, *IL-6* and *IFNγ*) in the lesional skin were also investigated. As a result, corticosteroid application suppressed the number of infiltrating inflammatory cells in the DD group of EB and SB at the early phase, and in DB at all time-points. However, the number of infiltrating inflammatory cells increased in EB on day 3. Expression of cytokines was generally suppressed in the PDN group of SB. In the cases of EB and DB, some cytokines had decreased but many of the others showed increased expression. In conclusion, the anti-inflammatory effects of corticosteroids are not simple inhibitory effects on inflammatory cell infiltration and cytokine production, but exert more complicated effects *in vivo*.

Keywords: *burn; corticosteroid; inflammatory cell infiltration; cytokine; mouse model*

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Introduction

Burns are one of the most common and devastating forms of trauma. For clinical applications, corticosteroid ointment is widely used for the initial care of a burn. The suppression of inflammation and accompanying pain and tissue damage is considered to be the theoretical basis of this application. However, no clear evidence has ever been reported. The healing of a skin wound is a systemic process consisting of three sequential phases including inflammation, proliferation and maturation. These phases are progressed by a complicated interaction of cells of various types[1]. A number of studies have demonstrated that a variety of cytokines, chemokines, growth factors and proteases are intimately involved in this process[1,2]. Therefore, it would provide useful information to clarify any alteration of lesional inflammatory cell infiltration and cytokine production in the inflammatory phase after corticosteroid application.

The aim of this study is to prepare mouse models of various grades of burn and, using these models, to evaluate the effects of corticosteroid application on each grade of burn in the acute inflammatory phase, especially on lesional infiltration of inflammatory cells and production of inflammatory cytokines.

Materials and Methods

Generation of mouse burn models

Hairless Hos:HR-1 mice were purchased from SLC (Hamamatsu, Japan). To generate three-graded burn models, hot water was applied with a cotton sponge on the back skin of adult (8 to 12 weeks old) female Hos:HR-1 mice[3]. To establish a model of epidermal burn (EB), dermal burn (DB) and subcutaneous burn (SB), hot water was applied at 70 °C for 1 s, at 80 °C for 5 s and at 80 °C for 10 s, respectively. To examine the effects of topical corticosteroid application, a strongest-class corticosteroid ointment (diflorasone diacetate, or DD) was applied on the back immediately after the hot water application in some mice. As a control, petrolatum was applied in some other mice. To examine the effects of systemic corticosteroid application, 1 mg/kg of prednisolone sodium succinate (PDN) was fed immediately after the hot water application in other mice (n = 10 in each group). All mice were fed with standard feed and given water *ad libitum*. This research protocol was approved by the Animals Care Committee of Wakayama Medical University.

Histological and immunohistochemical analyses

The mice were sacrificed 1–3 days after the hot water application, and the lesional skin samples were isolated and paraffin-embedded sections were made, followed by hematoxylin and eosin (HE) staining. In another series, some of them were immunostained with rabbit anti-mouse myeloperoxidase (MPO) polyclonal antibodies (pAb) (NeoMarkers, Fremont, CA) and rabbit anti-human CD3 pAbs (Dako Japan, Tokyo, Japan). The number of infiltrating inflammatory cells was enumerated using WinRoof® software (Mitani Corporation, Tokyo, Japan).

Quantitative analysis of local cytokine expression

The mRNA expression levels of inflammatory cytokines (*IL-1β*, *IL-6*, *TNFα* and *IFNγ*) in the lesional skin were investigated by real-time polymerase chain reaction (PCR) using SYBR Green Real-Time PCR System (Roche Diagnostics, Basel, Switzerland). *β-actin* was used for internal control. PCR primers were purchased from Nihon Gene Research Laboratories Inc. (Sendai, Japan). Each experiment was repeated three times and the mean ± standard errors of the mean (SEM) was shown.

Statistical analyses

Statistical analyses were performed with the Student's *t*-test.

Results

Generation of hot water-induced three grades of burn model on hairless mice

According to the protocol approved by the Animals Care Committee of Wakayama Medical University, hot water was applied on the back of hairless Hos:HR-1 mice to generate three grades of burn model. Hot water application at 70 °C for 1 s caused only EB, while DB or SB was stably developed when hot water was applied at 80 °C for 5 s or at 80 °C for 10 s, respectively (**Figure 1**).

Variable effects of corticosteroid application on burn grade-dependent inflammatory cell infiltration

On the histological analysis, the change in the number of infiltrating inflammatory cells was compared between control, DD and PDN groups. In general, the deeper the burn, the higher the number of infiltrating inflammatory cells were observed (EB < DB < SB), as shown in **Figure 2**. Since the ratio of the sum of MPO-positive and CD3-positive cells

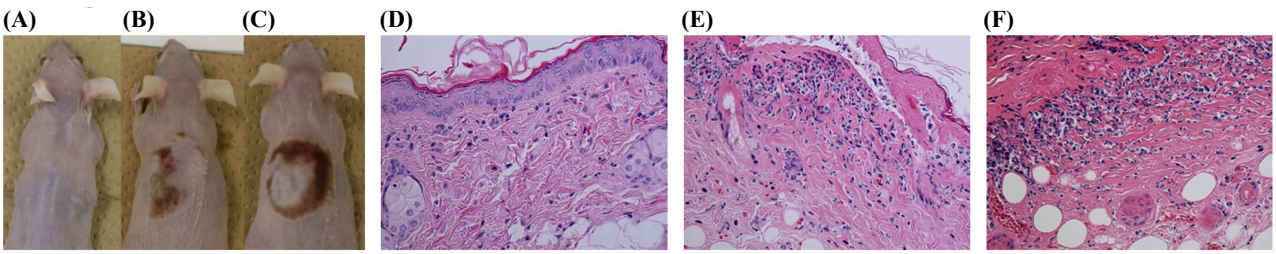

Figure 1. Gross and histological pictures of each degree of burn. Hot water was applied on hairless back skin of Hos:HR-1 mice to induce epidermal burn, or EB (**A**); dermal burn, or DB (**B**); and subcutaneous burn, or SB (**C**). Original magnification 200× of EB (**D**), DB (**E**), SB (**F**).

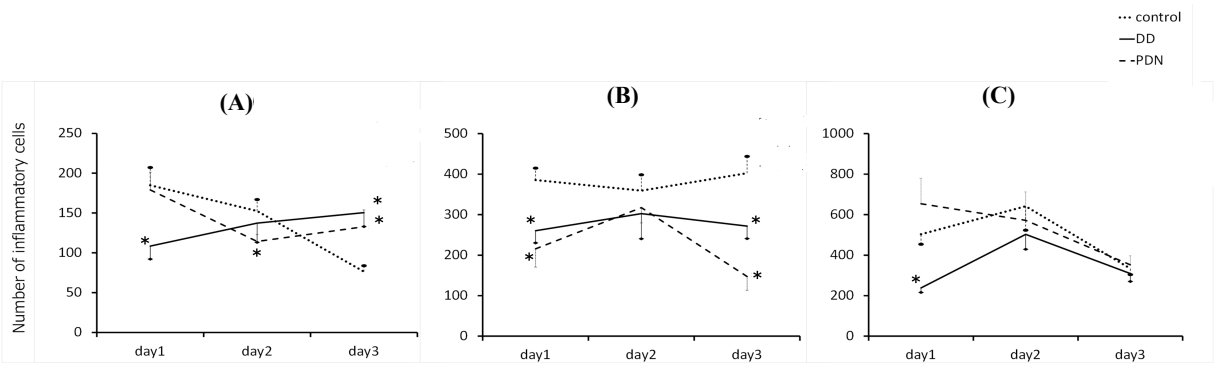

Figure 2. The number of infiltrating inflammatory cells in each degree of burn with/without corticosteroid application. After hot water-induced burn injury, lesional skin was isolated at indicated time-points and provided for hematoxylin and eosin staining. The dotted line, solid line and dashed line indicate control, corticosteroid (DD) and Prednisolone sodium succinate (PDN) groups, respectively. Statistical analyses were performed against the control group on each day. *$p < 0.05$, (**A**) epidermal burn, or EB, (**B**) dermal burn, or DB, (**C**) subcutaneous burn, or SB. Bars indicate standard errors of the mean (SEM).

in all infiltrating cells measured by HE stain reached almost 100% in EB, 95% in DB or 89% in SB (data not shown), most of the infiltrating inflammatory cells were neutrophils and lymphocytes.

In the case of EB, the number of infiltrating inflammatory cells was highest on day 1 and decreased for another two days in control mice. However, in the DD group, it was significantly lower on day 1 than in control and then increased for another two days to become significantly higher than the control on day 3 (**Figure 2A**). On the other hand, in the PDN group, it was almost the same level on day 1 but decreased to a significantly lower level on day 2 compared with the control, and then increased to a similar level as the DD group on day 3. These results suggest that inflammatory cell infiltration induced by EB was immediate and temporal (highest on or before day 1) event and topical corticosteroid application induced delayed and slower but sustained infiltration of inflammatory cells, while oral corticosteroid application showed delayed and only temporal inhibition of inflammatory cell infiltration.

In the case of DB, the number of infiltrating inflammatory cells in control mice reached almost twice that of EB on day 1 and remained at a similar level for another two days (**Figure 2B**). In the DD group, it was significantly lower than the control on day 1 and for another two days. In the PDN group, it was further decreased on days 1 and 3. These results suggest that DB-induced inflammatory cell infiltration was a prolonged (more than 3 days) event and topical and oral corticosteroid applications similarly showed stable inhibition of inflammatory cell infiltration.

In the case of SB, the maximum number of infiltrating inflammatory cells in control mice was observed on day 2 and was almost three times more than that of EB (**Figure 2C**). In the DD group, the number of infiltrating inflammatory cells was significantly lower only on day 1. In contrast, the number of infiltrating inflammatory cells in the PDN group was highest on day 1, without any significant difference from that in control at any time-point. These results suggest that inflammatory cell infiltration induced by SB was a delayed but temporal (highest on day 2) event and topical corticosteroid application showed immediate and only temporary inhibition of inflammatory cell infiltration, while oral corticosteroid application showed only small effects.

Variable effects of corticosteroid application on burn grade-dependent local inflammatory cytokine production

By quantitative PCR, the change in the lesional mRNA expression of inflammatory cytokines, including *IL-1β*, *TNFα*, *IL-6* and *IFNγ*, was compared between control, DD and PDN groups. In general, higher production of inflammatory cytokines was commonly observed in deeper burns (EB < DB < SB), as shown in **Figure 3**.

In the case of EB, weak production of IL-1β, TNFα and IFNγ, but almost no IL-6, was observed with a peak of IL-1β and IFNγ on day 2 and TNFα on day 1 in control mice (**Figure 3A–D**). IL-1β production was significantly lower in the DD group than in control on days 1 and 2, while it was higher on day 3 without significant difference. In the PDN group, it was higher on day 1 without significant difference, and significantly higher on days 2 and 3 (**Figure 3A**). TNFα production was significantly lower in the DD group than in control from day 1 to 3, while the same effect was observed only on day 2 in the PDN group (**Figure 3C**). Weak production of IL-6 was observed in both DD and PDN groups,

which was higher than in control on days 1 and 2 in the DD group and on day 3 in the PDN group without significant difference (**Figure 3B**). IFNγ production was significantly lower than in control on day 2 in the DD group. In contrast, it was higher than in control on days 1 and 3 in the PDN group without significant difference (**Figure 3D**).

To summarize, in our model of EB, weak mRNA expression of *TNFα* with following *IL-1β* and *IFNγ* was transiently induced. Most of the inflammatory cytokine production was significantly inhibited by topical corticosteroid, whereas significant up-regulation of IL-1β production on days 2 and 3 and the same tendency of other cytokines, except for inhibition of TNFα production on day 2, was observed after oral corticosteroid application.

In the case of DB, almost ten times more *IL-1β* and two times more *IL-6* and *IFNγ* mRNA expression was observed than in EB (**Figure 3A–H**). In control mice, mRNA expression of all four cytokines was only limited at all time-points, with an exception for much increased *TNFα* expression on day 3. IL-1β production was significantly higher than in control, on day 3 in the DD group and on days 2 and 3 in the PDN group (**Figure 3E**). IL-6 production also tended to be higher than in control on days 2 and 3 in both DD and PDN groups, whereas it was significantly lower on day 1 in the PDN group (**Figure 3F**). For TNFα and IFNγ production, the DD group showed almost the same level as the control at all time-points. In the PDN group, TNFα production gradually declined to a lower level than in control on day 3 without significant difference (**Figure 3G**). In contrast, IFNγ production in the PDN group was significantly higher on days 1 and 2, but had decreased to almost the same level on day 3, compared with that in control (**Figure 3H**).

To summarize, in our DB model, mRNA expression of *IL-1β*, *TNFα*, *IL-6* and *IFNγ* was quite limited, and only *TNFα* was significantly increased on day 3. Topical corticosteroid application showed almost no effect, except for significantly elevated production of IL-1β on day 3. For oral corticosteroid application, in addition to the similar effects, decreased TNFα production on day 3 and IL-6 production on day 1 and increased IFNγ production on days 1 and 2 were also observed .

In the case of SB, almost two times more IL-1β and IFNγ and four or five times more TNFα and IL-6 mRNA expression was observed than in DB (**Figure 3E–L**). In control mice, production of IL-1β, IL-6 and IFNγ peaked on day 1 and gradually decreased after that, while TNFα production peaked on day 2. In the DD group, IL-1β production tended to be lower on day 1, but higher on days 2 and 3 than in control (**Figure 3I**). In the PDN group, it was lower than in control for all three days. The same tendency was also observed for IL-6 and IFNγ production in the PDN group and for IL-6 production in the DD group (**Figures 3J and L**). TNFα production was significantly lower on day 2 in both DD and PDN groups, and tended to be lower on day 1 in the DD group and on day 3 in the PDN group, than in control (**Figure 3K**). In the PDN group, IFNγ production tended to be lower on day 1 than in control, while it gradually increased and tended to be higher on day 3 (**Figure 3L**).

To summarize, in our SB model, mRNA expression of *IL-1β*, *IL-6* and *IFNγ* with following *TNFα* was transiently induced. Oral corticosteroid application decreased any cytokine production at all time-points, whereas topical corticosteroid application increased IL-1β and IFNγ production on days 2 and 3.

Comparison of the effects of corticosteroid application on burn grade-dependent inflammatory cell infiltration and local cytokine production

To compare the variable effects of corticosteroid application more directly, the ratio of the number of infiltrating inflammatory cells in the DD group to control and that in the PDN group to control, as well as the ratio of relative mRNA expression level in the DD group to control and that in the PDN group to control for each cytokine, were calculated and were summarized in **Table 1**. Columns including the ratio <0.5 and >1.5 are highlighted in gray and black, respectively.

In the case of EB, the number of infiltrating inflammatory cells was lower than that of control on days 1 and 2, whereas it showed a greater than 1.5-fold increase over the control on day 3 in both DD and PDN groups. Similarly, lower expression on days 1 and 2 and higher expression on day 3 of *IL-1β* and *IFNγ* were observed in the DD group. In contrast, higher expression of *IL-1β* and *IFNγ* was observed throughout from day 1 to day 3 in the PDN group. *TNFα* expression was lower in DD, but was almost the same as in the PDN group, compared with control at all time-points. Interestingly, *IL-6*expression was always higher than control in both DD and PDN groups; it peaked on day 2 in DD and on day 3 in the PDN group.

In the case of DB, the number of inflammatory cells was always lower than control in both DD and

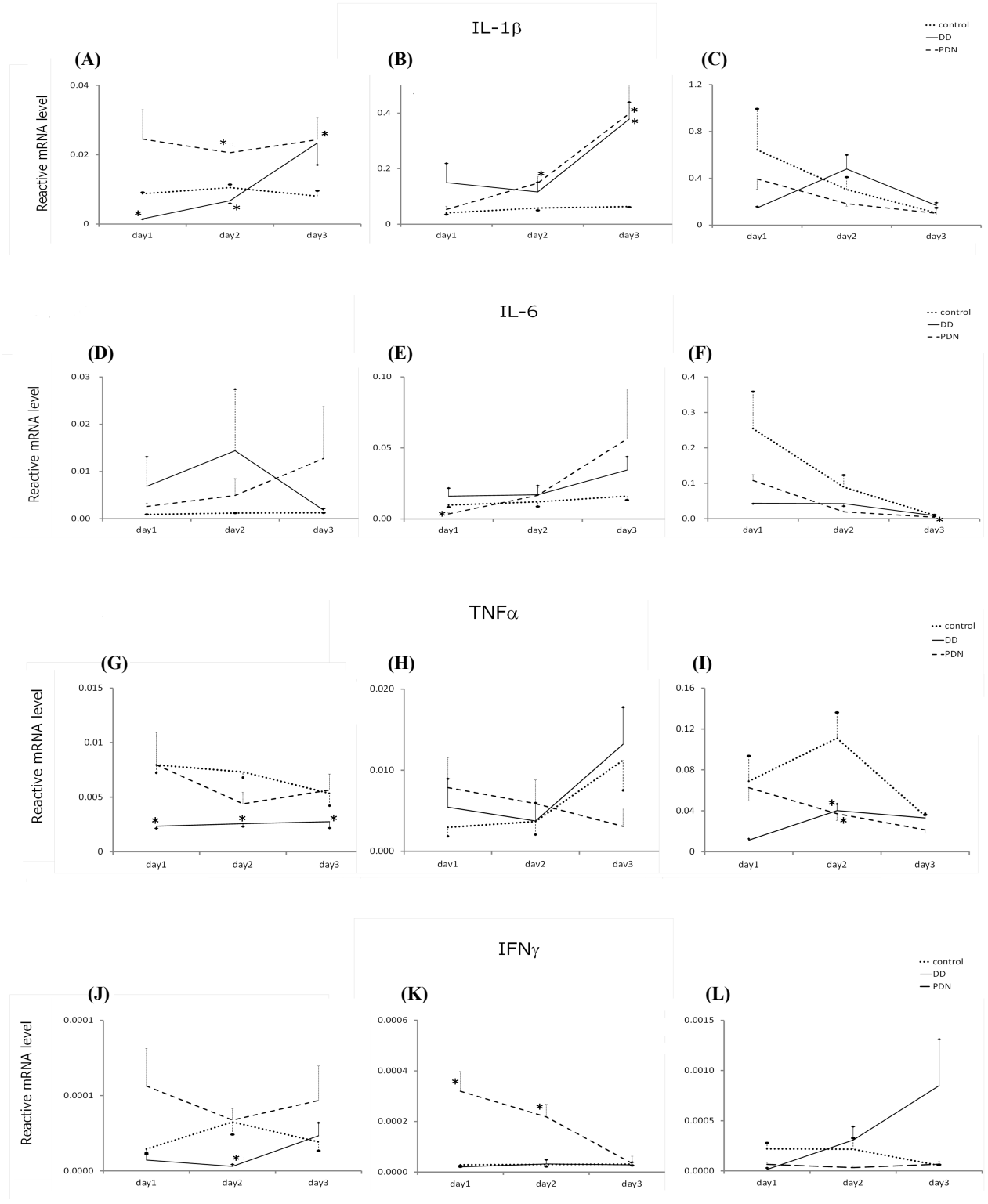

Figure 3. Local mRNA expression of inflammatory cytokines in each degree of burn with/without corticosteroid application. After hot water-induced burn injury, lesional skin was isolated at indicated time-points and provided for real-time PCR analysis. Relative mRNA expression levels were calculated using β-actin for internal control. The dotted line, solid line and dashed line indicate control, corticosteroid (DD) and Prednisolone sodium succinate (PDN) groups, respectively. *$p < 0.05$, (**A**) *IL-1β* on epidermal burn, or EB, (**B**) *IL-1β* on dermal burn, or DB, (**C**) *IL-1β* on subcutaneous burn, or SB, (**D**) *TNFα* on EB, (**E**) *TNFα* on DB, (**F**) *TNFα* on SB, (**G**) *IL-6* on EB, (**H**) *IL-6* on DB, (**I**) *IL-6* on SB, (**J**) *IFNγ* on EB, (**K**) *IFNγ* on DB, (**L**) *IFNγ* on SB. Bars indicate standard errors of the mean (SEM).

Table 1. The ratio of DD/control and PDN/control in the number of infiltrating inflammatory cells and relative mRNA expression levels of inflammatory cytokines in each degree of burn. The ratio of the number of infiltrating inflammatory cells in DD group to control and that in PDN group to control, as well as the ratio of relative mRNA expression level in DD group to control and that in PDN group to control for each cytokine, were calculated and summarized. Columns including the ratio <0.5 and >1.5 were highlighted in gray and black, respectively.

		Cells			*IL-1β*			*TNFα*			*IL-6*			*IFNγ*		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
EB	DD	0.59	0.90	1.97	0.17	0.64	2.91	0.29	0.35	0.51	7.59	12.09	1.45	0.49	0.09	1.21
	PDN	0.97	0.75	1.74	2.80	1.95	3.03	1.00	0.60	1.06	2.84	4.14	10.22	3.89	1.04	2.43
DB	DD	0.68	0.84	0.68	3.67	1.99	5.95	1.84	1.02	1.17	1.65	1.42	2.14	0.71	1.07	0.91
	PDN	0.56	0.88	0.37	1.30	2.55	6.26	2.65	1.60	0.27	0.36	1.38	3.51	11.28	7.27	1.11
SB	DD	0.47	0.78	0.93	0.23	1.58	1.59	0.16	0.36	0.95	0.17	0.48	0.88	0.07	1.42	15.93
	PDN	1.30	0.89	1.06	0.61	0.60	0.96	0.91	0.33	0.61	0.42	0.22	0.42	0.29	0.15	1.24

Note: EB: epidermal burn; DB: dermal burn; SB: subcutaneous burn; DD: corticosteroid; PDN: Prednisolone sodium succinate

PDN groups. In particular, it was lowest at 0.37 times in PDN on day 3. In contrast, significantly lower expression of cytokines was barely observed. Indeed, lower expression was observed only in cases with *IL-6* on day 1 and *TNFα* on day 3 in the PDN group.

In the case of SB, the number of inflammatory cells was lowest on day 1 and increased to be closer to the control on day 3 in the DD group. In the PDN group, it was higher than control on day 1, declined on day 2 and got closer to the control on day 3. In contrast, significantly higher expression of cytokines was barely observed. Indeed, higher expression was observed only in cases with *IL-1β* on days 2 and 3 and *IFNγ* on day 3 in the DD group.

In summary, applying corticosteroids for EB suppressed the number of infiltrating inflammatory cells at the early phase, but then markedly increased on day 3. The same tendency was observed for the expression of inflammatory cytokines, such as *IL-1β* and *IFNγ*, in the DD group, whereas in the PDN group most cytokines were expressed at higher levels than in control at all time-points. On the other hand, in the case of DB, coincidence of the suppression of inflammatory cell infiltration and the upregulation of cytokine expression was observed at all time-points, with only a few exceptions of the suppressed expression of *IL-6* on day 1 and *TNFα* on day 3 in the PDN group. Finally, in the case of SB, the number of inflammatory cells was suppressed in DD, but increased in the PDN group, at the early phase and then got closer to the control on day 3 in both groups. Although expression of cytokines was generally suppressed in the PDN group, higher expression of *IL-1β* on days 2 and 3, and much higher expression of *IFNγ* on day 3, were noted in the DD group.

Discussion

It has long been discussed, without conclusion, of how corticosteroids affect the initial treatment of burns and what kind of influence is exerted by corticosteroids on cytokine production and inflammatory cell infiltration. Furthermore, few data are available on the comparison of systemic corticosteroid application with its topical application for burn injury.

Corticosteroids are well known for their anti-inflammatory action. Burn injury causes direct destruction of epidermal and dermal tissue and accompanying increases in inflammatory cell infiltration and cytokine expression, resulting in swelling, pain and fever known as the inflammatory triad. Therefore, it is tempting to believe that suppression of such inflammatory reactions can be achieved by using corticosteroids. Actually, during clinical applications, it is sometimes noticed that corticosteroids seem to improve the burn-induced redness and swelling. Therefore, to clarify the therapeutic effects of corticosteroids on burn injury, we applied corticosteroids locally or systemically on mouse models of hot water-induced various grades of burn and examined the effect of corticosteroids on inflammatory cell infiltration and cytokine expression in their acute inflammatory stage.

On histological analysis, the number of infiltrating inflammatory cells was decreased in the case of the DB model. However, in the case of the EB and SB models, the results were not as expected. In particular, in the case of the EB model, the

number of inflammatory cells markedly increased on day 3 both in the DD and PDN groups. Since EB only causes transient redness without destruction of epidermal cells, the effect of burn injury was no longer expected on day 3. Although the precise cause of this result is unknown, the fact that a similar effect was observed for the expression of several cytokines in the DD group and, furthermore, most cytokines were always expressed at higher levels than in control in the PDN group, suggest the prolonged inflammation as an adverse effect of corticosteroid application on EB with least skin damage. In the case of the SB model, slightly increased numbers of infiltrating inflammatory cells were observed in the PDN group on day 1. However, for cytokine expression, no similar effect with rather general suppression by corticosteroid application was observed. Moreover, in spite of the decreased number of infiltrating inflammatory cells in the case of the DB model, increased expression of inflammatory cytokines was observed in most of the same specimens. These results indicate that corticosteroid application on burned skin does not simply decrease inflammatory responses, but rather induces more complicated responses dependent on the depth of burn injury, the method of steroid application and the period after burn.

A subtle spatiotemporal regulation of inflammatory and anti-inflammatory cytokine production is essential for the physiological process of wound healing[4]. Serum level of proinflammatory cytokines reflects the systemic response to burn injury and correlates with their local level, which directly reflects the local response to the burn. Proinflammatory cytokines such as IL-1α, IL-1β and TNFα, which belong to the first signaling molecules, were released by damaged keratinocytes and other resident cells in response to epidermal damage[5–7]. These cytokines induce the amplification circuit involving various cells. All of the locally produced cytokines contribute to the appearance of fever, production of acute-phase proteins, and an overall status of promoted catabolism. Furthermore, they upregulate the production of IL-6, prostaglandin E_2 (PGE_2) and platelet-activating factor by endothelial cells and macrophages[8,9].

Serum and local levels of IL-6 are increased after burn injury through its production by fibroblasts, macrophages, endothelial cells and keratinocytes. IL-6 has important functions in the proliferative phase of wound healing. Its downstream effects include indirect induction of neutrophil and macrophage infiltration, collagen deposition, angiogenesis, epidermal cell proliferation and tissue remodeling by the induction of tumor growth factor (TGF)-β1 and vascular endothelial growth factor (VEGF) production[10,11]. Actually, IL-6-deficient mice showed delayed wound healing because of reduced granulation tissue formation, re-epithelialization, angiogenesis, infiltration of macrophages and neutrophils, and matrix remodeling[12–14]. In burn patients, serum and local IL-6 levels peak approximately one week after injury, and its high level is reportedly associated with increased rates of morbidity and mortality[15]. IL-1β and TNFα, as well as IL-6, contribute to the following T cell activation through the production of acute-phase proteins[16].

IFNγ is another inflammatory cytokine produced by natural killer (NK) cells and T helper (Th)1 cells in response to injury and is different from the other three cytokines (IL-1, TNFα and IL-6)[17]. IFNγ has an important role in macrophage activation and differentiation of CD4 T cells into Th1 cells, with inhibition of their differentiation into Th2 cells[18]. Activated macrophages and Th1 cells are important in facilitating a proinflammatory response to injury and, hence, IFNγ is a cytokine related to the conversion from the inflammatory phase to proliferative phase of wound healing. A set of opposing cell types and cytokines can characterize an anti-inflammatory response and subsequent immunosuppression following burn injury[19]. Under specific circumstances such as after severe injury, inhibitory macrophages produce increased amounts of PGE_2 and decreased amounts of IL-12, which has a cooperative effect on Th1 cell differentiation[9,20,21]. Accordingly, CD4 T cells begin to differentiate into Th2 cells, which produce anti-inflammatory cytokines IL-4 and IL-10[18,22]. In particular, in the initial phase of burn, increased expression of IL-1, TNFα and IL-6 is induced in the Th1-dominant state. Subsequently, increased expression of anti-inflammatory cytokines is induced in the shifting from the Th1-dominant to the Th2-dominant state.

The imbalance of inflammatory and anti-inflammatory cytokine production can result in unbalanced Th1/Th2 polarity with hyper- or hypo-inflammation and, subsequently, in delayed wound healing. Indeed, significantly higher levels of IL-6 were detected in chronic long-lasting wounds than in acute rapidly-healing wounds[13]. This result suggests that the prolonged inflammation causes delayed wound healing in chronic wounds.

Takano and colleagues studied the time for 50% healing of wounds in rats which were given corticosteroid intramuscularly for three weeks pre-

and post-operatively, and found that the time was significantly shorter in the control group than in the corticosteroid-applied group[23]. In addition, lower expression of serum cytokines (IL-1β, IL-8, TGFβ and IFNγ) was observed in the corticosteroid-applied group on day 6. Therefore, long-term administration of corticosteroids may cause insufficient cytokine production in association with impaired wound healing.

Furthermore, corticosteroids generally decrease PGE_2 production and can be considered to have an influence on the secondary immunosuppression. An elevated level of glucocorticoids inhibits the production of IFNγ and IL-2, but not IL-4 and IL-10[9,24,25]. These effects can be another reason why corticosteroids cause a delay in wound healing[18,26].

Therefore, in our experiments, corticosteroid was applied only once immediately after the injury to avoid delayed wound healing. However, for cytokine production, clear anti-inflammatory effects of corticosteroids were observed only partly as expected, as follows: lower levels of IL-1β and IFNγ only in the early phase and a continuously lower level of TNFα in the DD group of the EB model; a lower level of IL-6 only in the initial phase and a lower level of TNFα only in the late phase in the PDN group of the DB model; a lower level of all four cytokines only in the early phase in the DD group of the SB model and continuously lower levels of IL-1β, TNFα and IL-6 and a lower level of IFNγ only in the early phase in the PDN group of the SB model. Nevertheless, higher expression of cytokines was noted on most of the other conditions.

Conclusion

These results indicate that corticosteroids do not simply inhibit inflammatory cell infiltration and cytokine production induced by burn injury even in its acute phase; rather, an alteration of the number of infiltrating inflammatory cells and expression of inflammatory cytokines is differentially determined by a set of burn grade and administration route of corticosteroids. Additionally, since lower inflammatory cytokine levels do not necessarily have better effects on wound healing, further studies are required for each application (DD/PDN group in EB/DB/SB model) to determine the precise mechanisms of the effects which corticosteroids exert on each phase of wound healing and to elucidate appropriate directions for the use of corticosteroids on therapeutics for burn injury.

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Conflict of Interest

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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ORIGINAL RESEARCH ARTICLE

Head-out immersion in hot water does not increase serum CXCL1 in healthy men

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ABSTRACT

Exercise-induced production of interleukin (IL)-6 results in the expression of chemokine CXC-motif ligand 1 (CXCL1) in mice. Recent studies described the increase in serum IL-6 levels during immersion of subjects in hot water. The present study investigated the effects of a 20-min head-out immersion in 42 °C water (hot-HOI) on serum concentrations of CXCL1 in eight healthy men. Venous bloods were taken at rest, immediately after hot-HOI, as well as 1, 2, 3, and 4 h after hot-HOI for measurements of serum concentrations of CXCL1, IL-6, tumor necrosis factor (TNF)-α, and high-sensitivity C-reactive protein (hsCRP), while assessing counts of blood cells (CBC) and monitoring core temperature (T_{core}). T_{core} and serum IL-6 increased during hot-HOI and remained high until 4 h after hot-HOI. However, serum CXCL1, TNF-α, hsCRP, and CBC remained constant throughout the experiment. In conclusion, the results from our study demonstrated that 20-min hot-HOI increased serum IL-6, but not CXCL1 in healthy men.

***Keywords**: IL-6; TNF-α; hsCRP; hyperthermia; myokine*

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Introduction

During systemic inflammatory state such as sepsis, serum levels of tumor necrosis factor (TNF)-α markedly increase just after an increase in serum interleukin (IL)-6. In contrast, the marked increase in serum level of IL-6 is not preceded by a rise in TNF-α during exercise[1]. Keller *et al.* suggested that the releasing of IL-6 from contracting skeletal-muscle fibers is mainly caused by *IL-6* gene transcription[2]. Therefore, IL-6 is considered a myokine[3,4]. Estimates from recent proteomic studies predict that the number of myokines would be more than 600 and belonging to different families[5].

What is the mechanism of exercise muscle-induced production of IL-6? While the exact stimulus is still being investigated, evidence suggests that local heating can act as a stimulus to express *IL-6* mRNA in skeletal muscle of mouse, *i.e.* the skeletal muscle acts as a "heat stress sensor"[6,7]. In addition, other studies showed that hot water immersion significantly increases IL-6 in humans, and concluded that skeletal muscles secrete IL-6 in response to exercise and heat exposure[8].

Human chemokine CXC-motif ligand 1 (CXCL1) is a 6.4-kDa polypeptide, classified as a chemokine that plays a role in recruiting leukocyte in inflammatory and immune reactions[9]. However, Nedachi *et al.* demonstrated that CXCL1 also works when released from contracting skeletal muscles[10]. Mice, in which the tibialis cranialis muscle over-expresses CXCL1, have lower visceral and subcutaneous fat mass, and the CXCL1-dependent decrease in adipose tissue mass coincides with improvements in glucose tolerance and insulin sensitivity in the whole

body[11]. Other studies have shown that hepatic expression of CXCL1 mediates the liver-protective potential of IL-6, and that CXCL1 is involved in wound healing, acts as a protectant against multiple sclerosis, and has potential neuroprotective effects[12-14].

The overexpression of IL-6 in murine muscles is followed by marked increase in serum level of CXCL1 and mRNA expression of *CXCL1* in the liver[15]. In an experimental study involving wild-type mice, 1 h of swimming exercise induced increases in serum IL-6 immediately after exercise, and in serum CXCL1 2 h after exercise, whereas the same exercise did not modulate liver *CXCL1* mRNA expression in IL-6 knockout mice[15]. The results suggest a tight muscle–liver crosstalk during exercise, in which exercise-induced IL-6 induces a production of CXCL1 in the liver[15].

Based on the above findings, we hypothesized that heat stress-induced IL-6 increases CXCL1 expression in humans. To test our hypothesis, we measured in the present study serum CXCL1 levels before, during and after 20-min head-out immersion in 42 °C water (hot-HOI) in a group of normal adults.

Materials and methods

Subjects

Eight healthy males voluntarily participated in the present study. **Table 1** lists the characteristics of these subjects. None of the subjects were on any medications at the time of the study.

Table 1. Characteristics of the eight participating subjects

Variable	Healthy males (mean ± SD)
Age (years)	25.9 ± 3.5
Height (cm)	173.3 ± 4.0
Weight (kg)	70.1 ± 12.0
BMI (kg/m^2)	23.3 ± 3.6

Experimental protocol

The subjects were informed to refrain from strenuous physical activity and alcohol on the day before the study and to refrain from taking any fluids or foods from 2200 h the night before the study, except for tap water, until the completion of the study. They reported to the laboratory at 0800 h on the experiment day. Each subject wore a swimming trunk at the time of the study. A copper-constantan thermocouple probe was inserted into the esophagus and its tip placed at the atrium level to monitor body core temperature (T_{core}) throughout the study. A heparinized indwelling catheter was placed into the right antecubital vein. Before the onset of the study, the subjects sat in a room outside of the water tank for 1 h as the control period. After confirmation of stable T_{core}, measurements were started during the 10-min rest in the sitting position outside the tank. Then, the subject walked into and sat in the hot (42 °C) water tank, immersing the entire body, except the head, into the water. Measurements were taken after 20 min, then the subject left the tank to sit nearby for 4 h and measurements were repeated during this period. Thus, the entire measurement was of 270 min duration. Venous blood samples were collected just before hot-HOI (pre-HOI), 30 min (at end of HOI), 90 min (1 h post-HOI), 150 min (2 h post-HOI), 210 min (3 h post-HOI), and 270 min (last measurement) from the onset of the study. Blood samples (6 mL each) were withdrawn through the intravascular catheter and were used for the measurements of IL-6, CXCL1, TNF-α, high sensitivity C-reactive protein (hsCRP), hematocrit, and counts of blood cells.

Analysis of blood samples

Total blood cell counts were assessed by using a cell counter. Hematocrit was determined by centrifugation. Other venous blood samples were drawn into pre-chilled serum venipuncture tubes. The tubes were centrifuged at 3500 rpm for 10 min at 4 °C, and then the serum was separated and stored at −80 °C until analysis. IL-6 was measured by enzyme immunoassay (ELISA) for IL-6 (R&D Systems, Minneapolis, USA) with assay sensitivity of 0.039 pg/mL and intra- and inter-assay coefficients of variability (average CV of different concentrations) of 7.4% and 7.8%, respectively. CXCL1 was assayed using ELISA for CXCL1 (R&D Systems) with assay sensitivity of <10 pg/mL and intra- and inter-assay coefficients of variability of 2.9% and 5.2%, respectively. TNF-α was analyzed using ELISA for TNF-α (R&D Systems) with assay sensitivity of 0.106 pg/mL and intra- and inter-assay coefficients of variability of 5.4% and 8.3%, respectively. IL-6, CXCL1, and TNF-α immunoassays were performed in duplicate by investigators blinded to the study design.

Statistical analysis

All results were represented as mean ± SEM except when noted otherwise. The differences between two parameters were compared with one-way analysis of variance, followed by multiple

comparisons at various time points. When the *F*-value was significant ($p < 0.05$), the time period and the study condition comparisons were made using Fisher's LSD test.

Ethics statement

The study protocol was approved by the Ethics Review Committee of Wakayama Medical University and conformed to the Declaration of Helsinki. A signed informed consent was obtained from each subject after a complete explanation of the purpose and risks of the present study.

Results

T_{core} significantly increased immediately after HOI, and was still higher at 1 h post-HOI, 2 h post-HOI, and 3 h post-HOI, but recovered to pre-HOI on the last measurement (**Figure 1**). **Table 2** lists the blood cell counts at the six different time points. Hemoglobin, hematocrit, erythrocyte count, and monocyte count remained constant throughout the study. Serum IL-6 concentrations were significantly higher at 1 h post-HOI, 2 h post-HOI, and 3 h post-HOI, and recovered to pre-HOI level in the last measurement (**Figure 2A**). Serum concentrations of CXCL1 (**Figure 2B**), TNF-α (**Figure 3A**), and hsCRP (**Figure 3B**) remained unchanged throughout the experiment.

Discussion

This is the first study that examines the serum level of CXCL1 to hot-HOI in young and healthy men. The major findings of this study are the followings: 1) hot-HOI resulted in increased levels of serum IL-6; 2) no changes in serum CXCL1 levels were noted; and 3) the levels of T_{core} and IL-6 returned to baseline at 4 h after hot-HOI. Thus, hot-HOI activated the releasing of IL-6 but did not stimulate the releasing of CXCL1. The increase in serum IL-6 was not induced by dehydration during hot-HOI because hematocrit and erythrocyte count did not change after hot-HOI, compared with the baseline.

It is well known that expressions of mRNA for *IL-6* and other proinflammatory cytokines, such as *TNF-α* and *IL-β*, are mainly regulated by the toll-like receptor (TLR) signaling cascade, which results in nuclear translocation and activation of

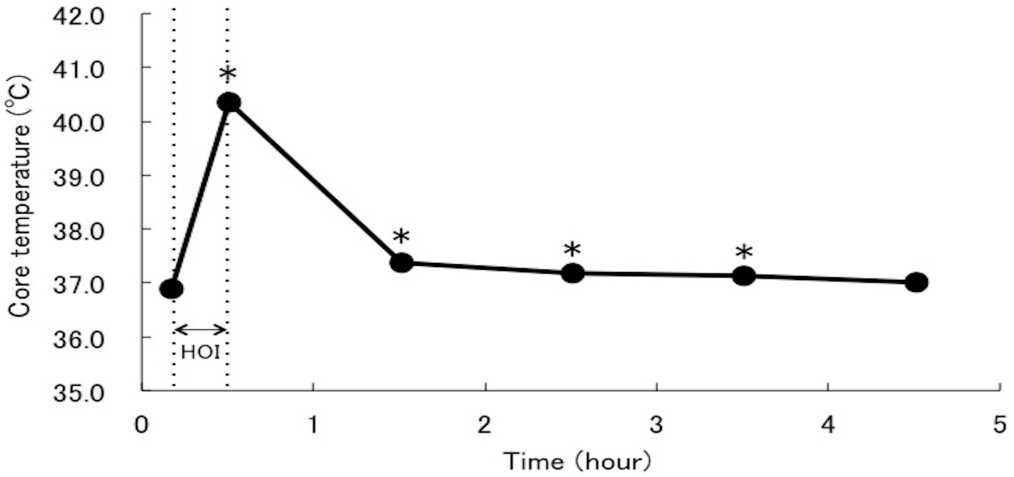

Figure 1. Core temperatures before and after head-out immersion (HOI) in hot water (42 °C). The left side of the plot represents data before HOI. Time 0 represents the onset of the study. The time between the two vertical dashed lines represents the time spent in hot-HOI. Data are mean ± SEM. *$p < 0.05$, compared with before immersion.

Table 2. Hemoglobin, hematocrit and blood cell count values measured during the study

	Pre-HOI	Immediately after immersion	After immersion			
			1 h post-HOI	2 h post-HOI	3 h post-HOI	4 h post-HOI
Hemoglobin concentration (g/dL)	15.0±0.3	15.4±0.3	15.4±0.3	15.0±0.5	15.5±0.3	15.5±0.2
Hematocrit (%)	44.2±0.8	45.6±0.9	45.7±0.8	44.4±1.3	45.9±0.9	45.5±0.6
Erythrocyte count (×10^5/μL)	49.2±1.1	50.7±1.2	50.9±1.2	49.4±1.6	51.2±1.2	50.6±0.9
Monocyte count (10^2/μL)	1.9±0.4	1.4±0.2	1.8±0.3	1.9±0.3	2.1±0.2	1.9±0.2

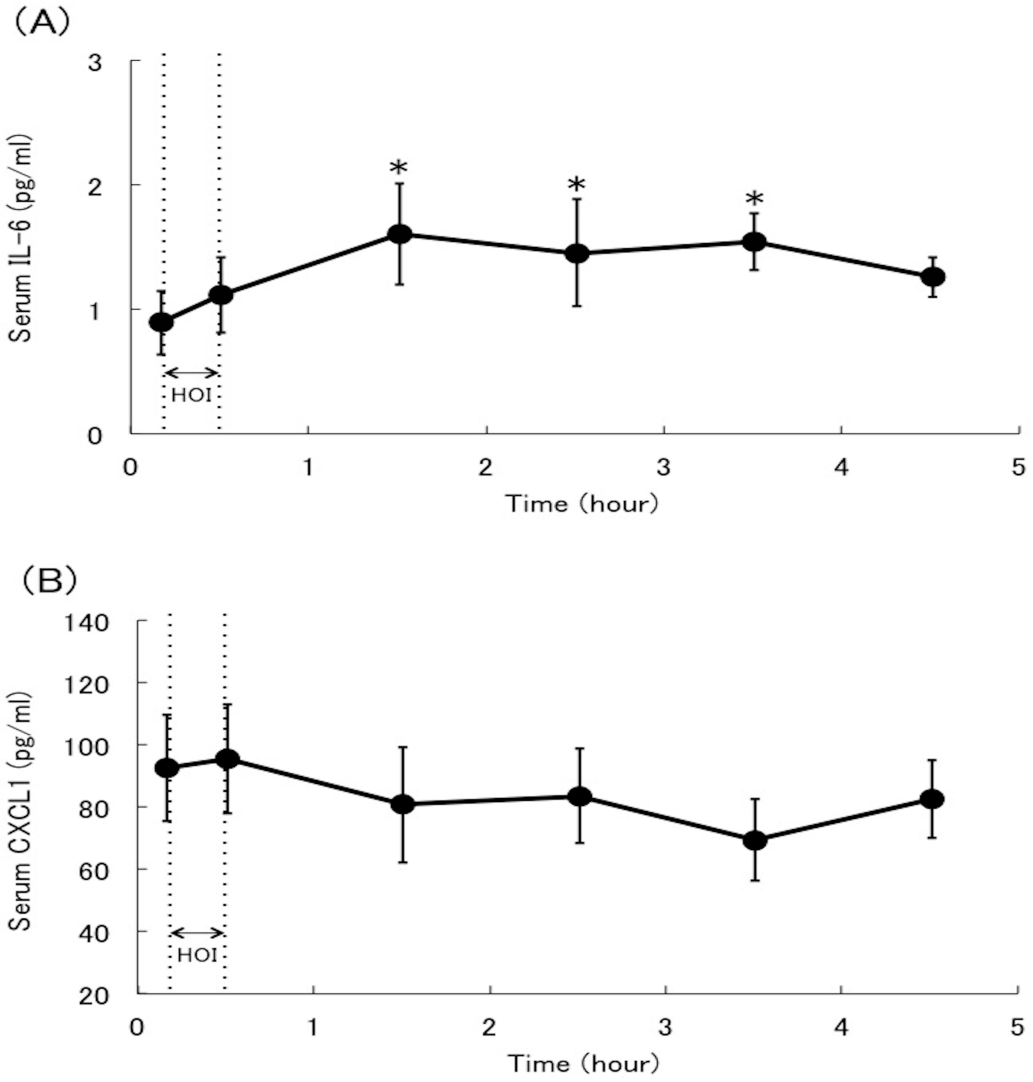

Figure 2. Serum IL-6 (**A**) and CXCL1 (**B**) levels measured before and after head-out immersion (HOI) in hot water (42 °C). The left side of the plot represents data before HOI. Time 0 represents the onset of the study. The time between the two vertical dashed lines represents the time spent in hot-HOI. Data are mean ± SEM. *$p < 0.05$, compared with before immersion.

NF-κB during systemic inflammatory state such as sepsis. In contrast, exercise-induced increase in serum IL-6 appears in advance of a rise in TNF-α[1]. The stable levels of TNF-α, hsCRP, and monocyte count throughout the present measurement indicate the lack of inflammatory response during hot-HOI. Therefore, the increase in serum IL-6 is unlikely to be induced through TLR receptor signaling cascade. On the other hand, the simultaneous recovery of T_{core} and IL-6 strongly suggests that T_{core} is a strong activator for releasing IL-6.

Previous studies demonstrated the interactions between T_{core} and IL-6 production during exercise. Rhind *et al.* reported that the decrease in T_{core} during endurance exercise is associated with attenuation of the IL-6 response[16]. Starkie *et al.* reported the augmentation of the circulating IL-6 response when endurance exercise is performed in a hot environment, and concluded that releasing IL-6 from contracting skeletal muscles is a temperature-related phenomenon[17]. Another study described the upregulation of IL-6 in skeletal muscle following exposure to heat, both *in vitro* and *in vivo*[6]. Our results of changes in IL-6 level after hot-HOI strongly suggest that the increase in T_{core} is an independent factor responsible for the increase in serum IL-6. The significant rise in IL-6 level after hot-HOI corresponds to the findings of previous study[8]. Interestingly, hyperthermia is reported to induce the release of IL-6 from skeletal muscle tubules in mice[6]. Considered together, it seems that

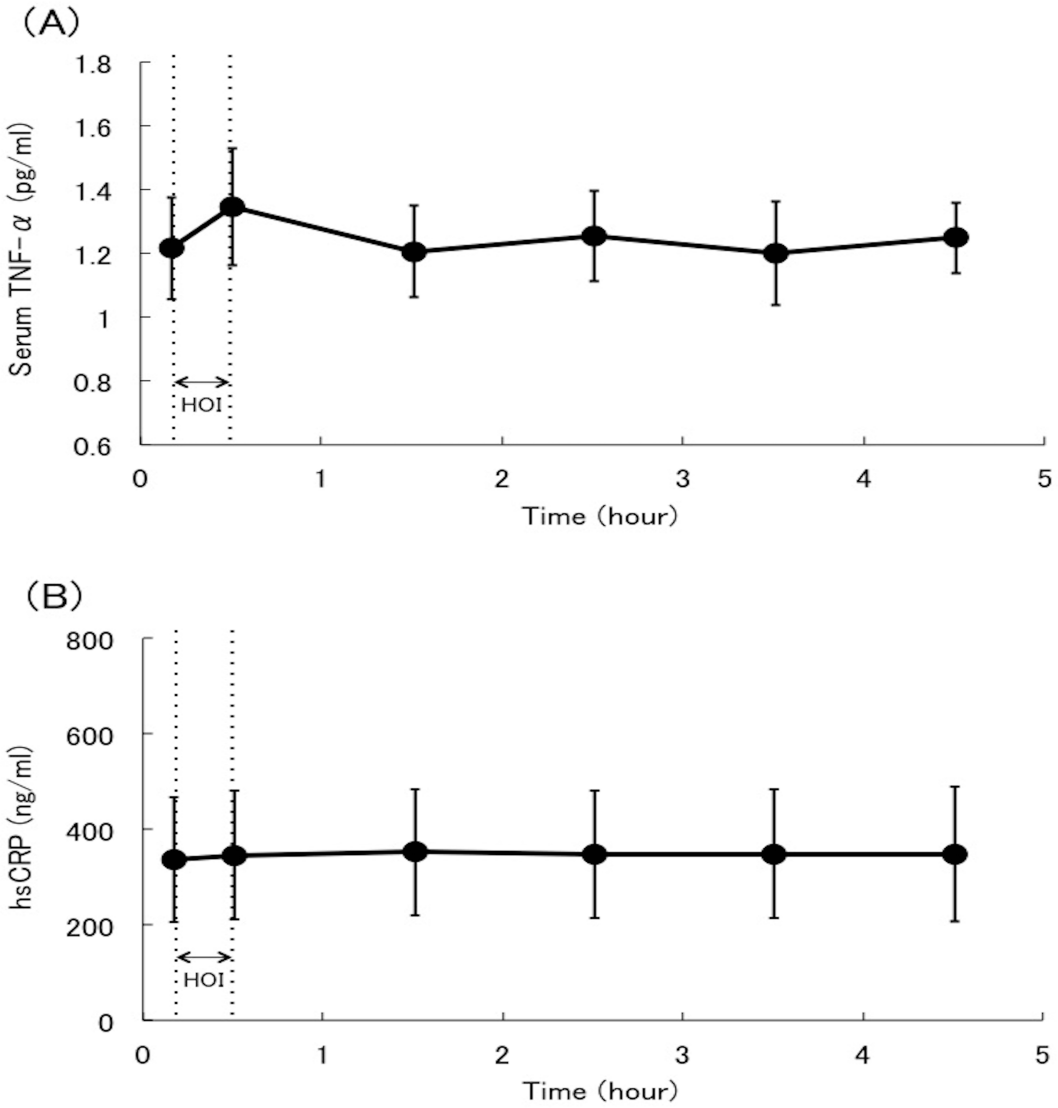

Figure 3. Serum TNF-α (**A**) and hsCRP (**B**) levels before and after head-out immersion (HOI) in hot water (42 °C). The left side of the plot represents data before HOI. Time 0 represents the onset of the study. The time between the two vertical dashed lines represents the time spent in hot-HOI. Data are mean ± SEM.

skeletal muscles are one of the organs which produce IL-6 at least after hot-HOI.

Serum CXCL1 protein levels and *CXCL1* mRNA expression levels in muscle and liver increase in swimming mice[15]. Importantly, the induction of CXCL1 expression is highest in the liver; the liver being the main source of releasing CXCL1 after exercise[15]. The same study demonstrated that IL-6 is released from the muscle-regulated liver CXCL1 expression[15]. One hour of swimming exercise would induce a 5-fold increase in serum IL-6 in wild-type mice immediately after exercise, and the increase was still seen at 2 h post-exercise, which was coupled with an increase in serum CXCL1[15]. In another study of mice, 30 min of treadmill running induced a 2.8-fold increase in serum IL-6, together with an increase in serum CXCL1[10]. In the present study, hot-HOI induced a 2-fold increase in serum IL-6 but no change in CXCL1. Therefore, the stability of CXCL1 and increase in IL-6 level suggest that hyperthermia *per se* contributed to the decrease in CXCL1 expression.

Previous studies described a close relationship between increased expression of *IL-6* mRNA in the skeletal muscle in a hot environment and those of heat shock protein *(HSP)-72* mRNA, and that treatment with KNK437, a heat shock factor (HSF) inhibitor, significantly reduced *IL-6* mRNA expression[6]. The main pathway involved in regulation of releasing IL-6 in various stressed

conditions, including hyperthermia, activates mitogen-activated protein kinases (MAPK), or more specifically, stress-activated protein kinases (SAPK) such as c-jun N-terminal kinase (JNK)[18]. JNK increases a transcription of IL-6 through the downstream activation of c-fos and c-jun, which dimerize and bind to the activator protein-1 (AP-1) regulatory element on the IL-6 promoter. Welc *et al.* examined the heat-induced transcriptional control of IL-6 in C2C12 muscle fibers, and demonstrated that the regulation of releasing IL-6 in hyperthermia is directly controlled by HSF-1 and AP-1 signaling[7]. Nedachi *et al.* have recently succeeded in establishing an advanced *in vitro* muscle exercise model using highly developed C2C12 myotubes that possess electric pulse stimulation (EPS)-evoked vigorous contractile activity[10]. They found marked upregulation of CXCL1 expression in contractile C2C12 myotubes after 24-h EPS. Moreover, they strongly suggested that the NF-κB signaling pathway is directly involved in CXCL1 expression in response to EPS-evoked contractility[19]. However, NF-κB signaling was reported to be downregulated by hyperthermia in mouse myoblast cells[20]. These results of the above studies might explain the lack of change in serum CXCL1 level and the increase in serum IL-6 level after hot-HOI.

The other possible explanation for the lack of change in serum CXCL1 level after hot-HOI would be related to an insufficient expression level of IL-6; even serum IL-6 significantly increased after hot-HOI. Furthermore, there was no contraction of skeletal muscles and/or lower energy expenditure because sitting would be associated with the lack of altering in the serum CXCL1 level[15]. However, it is not clear the reason why serum CXCL1 remained unchanged after hot-HOI.

Conclusion

The present study demonstrated that 20 min of hot-HOI did not affect CXCL1 expression in young healthy men, but increased serum IL-6 levels, probably through the increase in core body temperature.

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Conflict of interest

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of their article.

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ORIGINAL RESEARCH ARTICLE

Keigairengyoto, a traditional Japanese medicine, promotes bacterial clearance by activating innate immune cells in mouse cutaneous infection models

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ABSTRACT

Prompt elimination of pathogens including bacteria and dead cells prevents the expansion of secondary and prolonged inflammations and tissue damage. Keigairengyoto (KRT) is a traditional Japanese medicine prescribed for dermatoses such as purulent inflammations. Our aim is to clarify the actions of KRT in bacterial clearance and to examine the cell-kinetic profiles of phagocytes. In a mouse cutaneous infection model using living *Staphylococcus aureus*, KRT drastically reduced the number of bacteria in the infection sites. To evaluate the bacterial clearance, pseudo-infection was induced in mouse ears by intradermal injection of FITC-conjugated dead S. *aureus*. Biochemical and histological examinations revealed that KRT promoted bacterial clearance at 6 and 24 h post-injection. The numbers and phagocytic activities of neutrophils and macrophages in the ears were evaluated histologically using anti-Ly6G and F4/80 antibodies. KRT reduced bacterial deposition and increased the accumulation of F4/80$^+$ resident macrophages around the lesion site. FACS analysis was performed on single cell suspensions dispersed enzymatically from skin lesions, followed by an investigation of CD11b$^+$Ly6G$^+$ (neutrophils) and CD11b$^+$Ly6G$^-$ (monocytes/macrophages) cells. KRT increased the mean fluorescent intensity of FITC in CD11b$^+$Ly6G$^-$ cells and the number of FITC-positive CD11b$^+$Ly6G$^+$ cells, while KRT did not change the numbers of these cells. To investigate the active constituents of KRT, phagocytosis assay using macrophages was performed, resulting in that some flavonoid glucuronides of KRT derivatives augmented phagocytosis. Collectively, KRT promoted bacterial clearance by enhancing the phagocytic capability of neutrophils and macrophages. KRT may exert unique properties in preventive and therapeutic strategies for skin infectious inflammation.

Keywords: *kampo; phagocytosis; resident macrophages; neutrophils; Staphylococcus aureus; flavonoid glucuronide; skin*

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Introduction

The skin functions as a barrier to pathogens such as bacteria. In injured skin or immunosuppressive conditions, bacteria can enter the body, leading to infectious inflammation. Chronic stimulation by bacteria can cause skin inflammation, and dysregulated responses to infection can induce life-threatening septic shock. Innate immune cells possess receptors for pathogen-associated molecular patterns (PAMPs) and eliminate foreign invaders[1]. Prompt, flexible, and appropriate clearance of PAMPs is important to prevent the expansion of excessive inflammatory reactions and prolonged inflammations.

Professional phagocytes, such as neutrophils, macrophages, and dendritic cells, play roles to eliminate harmful and/or inflammatory particles. Circulating neutrophils are rapidly recruited to infectious areas through chemotactic signals[2]. Neutrophils dominantly express various receptors for PAMPs and bear the primary defense against infection by killing bacteria through some bactericidal mechanisms[3]. Macrophages and dendritic cells are residents in the skin and function as sentinel cells in the body's first line of defense against microbes as well as neutrophils[4,5]. Macrophages have various types of receptors

for PAMPs and manage the innate immune system, while dendritic cells are the key cells in the development of acquired immune system as antigen-presenting cells. Macrophages have characteristics of diversity and plasticity in cell differentiation, therefore playing roles in inflammation and tissue repair and regeneration[6]. Resident dermal macrophages orchestrate the initiation and termination of immune response in skin infection[7]. A deficiency of macrophage function fails in the timely recruitment of neutrophils to the site of infection and delays bacterial clearance[7]. These lines indicate that resident dermal macrophages play important roles in the initial detection of skin-infiltrating bacteria and the activation of neutrophils.

Keigairengyoto (KRT) is a pharmaceutical grade traditional Japanese (*kampo*) medicine that is prescribed to patients with purulent dermatoses, which generate many dead neutrophils, followed by non-infectious inflammation. KRT is also prescribed to ameliorate purulent inflammation in other organs, such as empyema and rhinitis. KRT comprises 17 crude drugs and abundantly includes flavonoids, some of which enhance the phagocytic activity of myeloid leukemia cells and activate macrophage-like cells[8-12]. Genistein, which is rich in Glycyrrhizae radix, is reported to modify macrophage differentiation[13,14], inhibit LPS-induced downregulation of monocyte chemotactic protein-1(MCP-1) receptors, and promote the potentials of skin tissue repair[15-17]. Given that KRT is clinically effective against purulent inflammation in humans, these reports suggest that KRT enhances macrophage function. We therefore hypothesized that KRT activates innate immune response and promotes elimination of bacteria. As the skin resident bacterium *Staphylococcus aureus* is involved in skin diseases, such as impetigo and cellulitis, we evaluated the effects of KRT in mouse infection models using *S. aureus*. As expected, KRT markedly reduced the number of living *S. aureus* in the site of infection. We also examined the cell-kinetic profiles of neutrophils and macrophages in a pseudo-infection model using FITC-conjugated dead *S. aureus* and we observed KRT-enhanced bacterial clearance through modification of the immune system.

Materials and methods

Test drugs

KRT was supplied by Tsumura & Co. (Tokyo, Japan) in the form of a powdered extract. It was obtained by spray-drying a hot-water extract mixture of the following 17 crude drugs: Scutellariae radix, Phellodendri cortex, Coptidisrhizoma, Platycodi radix, Aurantiifructusimmaturus, Schizonepetaespica, Bupleuri radix, Gardeniaefructus, Rehmanniae radix, Paeoniae radix, Cnidiirhizoma, Angelicae radix, Menthaeherba, Angelicaedahuricae radix, Saposhnikoviae radix, Forsythiaefructus, and Glycyrrhizae radix.

Many papers demonstrated that the commonly used and effective dose of *kampo* medicines is approximately 1 g/kg body weight in animal experiments including in dermatitis study[18,19]. Plasma pharmacokinetic study of bioactive flavonoids, which are speculated as active constituents of KRT, showed that they are absorbed promptly and transiently into the systemic circulation[18]. In order to examine the preventive effect of KRT, we administrated KRT at 0.25–2 g/kg body weight 1 h before and after cutaneous stimulation.

Prednisolone sodium succinate (PDN) was purchased from Shionogi Pharmaceutical Co. (Osaka, Japan) and used as a reference drug. Genistein 7-*O*-glucuronide and hesperetin 7-*O*-glucuronide were purchased from Toronto Research Chemical Industries (Toronto, ON, Canada). Liquiritigenin 4'-*O*-glucuronide, liquiritigenin 7-*O*-glucuronide, 18β-glycyrrhetinic acid, and cimifugin with purities high enough to be evaluated in biological tests were obtained from Analytical and Pharmaceutical Technology Research Center, Tsumura & Co. These compounds are absorbed through the digestive system and detected in the systemic circulation of humans and rats[18,20].

Animals

Male BALB/c and ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and Charles River Laboratories Japan Inc. (Kanagawa, Japan), respectively. Each mouse was used at 8–10 weeks old. This study was approved by and conducted according to the guidelines of the Experimental Animal Ethics Committees of Tsumura & Co.

Superficial skin infection model

Living S. aureus-induced cutaneous infection was performed in BALB/c mice according to the procedure described by Kugelberg *et al.* with a minor modification[21]. The hair on the back was shaved with an electric razor and depilatory cream under ketamine anesthesia was applied. An area of approximately 4 cm^2 was stripped with cloth-made cohesive tape once a day for two days. After the stripping in the second day, *S. aureus* MW2 (strain: BAA-1707, ATCC, Manassas, VA, USA)

was inoculated on the skin at 1×10^7 colony forming unit (CFU) in 100 µL saline. KRT was administrated orally to the mice at 1 and 2 g/kg in distilled water 1 h before and 1, 2, and 3 days after the inoculation. Skin lesion was examined at days 2 and 4 after the inoculation. The severities of erosion and papule were scored according to the following criteria: 0 (none), 1 (mild), 2 (moderate), and 3 (severe), and were indicated as the sum of each score. Mice were sacrificed by exsanguination under anesthesia at day 4 after the inoculation, and the skin of the infected site was excised and homogenized in saline. The homogenates were plated on staphylococcus agar to determine the number of living bacteria. The number of living bacteria was indicated as CFU per weight of the skin tissues (CFU/g).

Pseudo-infection model

Pseudo-infection model was performed in ICR mice using FITC-conjugated bioparticles of heat- or chemically-killed *S. aureus*, which were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). FITC-conjugated *S. aureus* (2×10^7 particles in 10 µL saline) or saline alone was intradermally injected into the ventral side of both ears using a microsyringe (Hamilton Co., Reno, NV, USA) under isoflurane anesthesia. KRT was administrated orally to the mice at 0.25 and 1 g/kg in distilled water 1 h before and 6 h after the injection. PDN was administrated orally to the mice at a dose of 10 mg/kg in distilled water. The mice were sacrificed by exsanguination under anesthesia 2, 6, or 24 h after the injection, and the ears were cut off for the following examinations.

Measurement of bacterial clearance

Bacterial clearance from the infected ears was evaluated by two methods. First, the ears were immersed in 1 N KOH and lysed completely, followed by the measurement of fluorescent intensity at an excitation wavelength of 495 nm and an emission wavelength of 528 nm. The amount of bacteria was calculated using a standard curve of serially diluted FITC-conjugated *S. aureus*. In the second method, the ears were stained by Gram's method as described in the next section, and the depositions of Gram-stained bacteria were semi-quantified by image analysis using BZ-X analyzer software (Keyence, Osaka, Japan).

Histological evaluation

The back skin and the ears were fixed with 10% formaldehyde and 4% paraformaldehyde, respectively. The fixed tissues were embedded in paraffin and cut into 4-µm cross-sections. The specimens were stained with hematoxylin and eosin (HE) or Gram-Hacker's solution. For immunofluorescent analysis, sections were stained using 2 µg/mL of anti-mouse F4/80 (clone: A3-1, Bio-Rad Laboratories Inc., Hercules, CA, USA) and 1 µg/mL of Alexa 647-conjugated anti-rat IgG2b (Thermo Fisher Scientific Inc.), or 2 µg/mL of biotin-conjugated anti-mouse Ly6G (clone: 1A8, BioLegend, San Diego, CA, USA) and 0.1 µg/mL of APC-conjugated streptavidin (BioLegend), and then mounted using ProLong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific). All images were taken using a Biorevo BZ-X700 fluorescence microscope and BZ-X viewer software (Keyence). The accumulation of F4/80-positive cells in the inflamed ears was evaluated as the number of positive cells per unit area within the range of 200 µm around the clumps of bacteria and/or abscesses. F4/80-positive cells were identified according to the double-positive stain of Alexa647 and DAPI.

Measurement of chemokines and cytokines in inflamed ears

The ears obtained at 1 h after the *S. aureus* injection were frozen immediately in liquid nitrogen, and then were crushed using a cryopress machine (Microtec Co., Chiba, Japan). The crushed ears were homogenized in cold PBS supplemented with a cocktail of proteinase inhibitors (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 10,000 g for 15 min at 4 °C. MCP-1, interleukin-1β (IL-1β), and keratinocyte chemoattractant (KC) in the supernatant of homogenates were quantified using ELISA kits according to the manufacturer's instructions (R&D Biosystems, Minneapolis, MN, USA). Total protein was measured by the Lowry assay (Thermo Fisher Science) using bovine serum albumin as a standard.

Immune cell analysis by flow cytometry

The procedure was performed according to the previous report with a minor modification[7]. The ear was cut into nine pieces, followed by enzymatic digestion in PBS containing 0.45 mg/mL dispase I (Roche Diagnostics, Indianapolis, IN, USA), 2 mg/mL collagenase II (Sigma-Aldrich), and 0.32 mg/mL DNase I (Sigma-Aldrich) for 2 h while shaking at 37 °C. Single-cell suspensions were prepared by mechanical disruption in ice-cold Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, followed by filtration through a 70-µm cell strainer. After centrifugation twice, the cells were gently suspended in 30% Percoll (GE Healthcare, Piscataway, NJ, USA),

and then centrifuged at 630 g for 20 min to isolate and remove sebum, low-density cells and debris. Cell pellets were re-suspended in 10% FBS DMEM and passed through a 40-μm cell strainer. After centrifugation, the cells were fixed with paraformaldehyde and stored at 4 °C until flow cytometric analysis. After blocking non-specific binding using mouse IgG1 (clone MG1-45, BioLegend), the following antibodies to mouse antigens were used: PE anti-mouse CD45 (clone: 30-F11, BD Bioscience, San Diego, CA, USA), PE/Cy7 anti-CD11b (clone: 30-F11, BioLegend), and biotin anti-Ly6G (clone: 1A8, BioLegend). APC-conjugated streptavidin was used for the secondary reagents. Cell samples were analyzed with a two-laser flow cytometer FACSaria II, and the data were analyzed with Diva software version 8.0.1 provided by Becton, Dickinson & Company (San Jose, CA, USA). Debris (FCS *vs.* SSC) and doublets (FSC-H *vs.* FSC-A) were excluded. Cells from the ears of a naive mouse showed approximately 15% CD45-positive and approximately 8% CD11b-positive cell populations in the whole cells. Phagocytic activity was shown as the mean fluorescence intensity (MFI) of whole cells.

Measurement of ear thickness

Ear thickness was measured using a dial thickness gauge micrometer (Ozaki MFG Co., Tokyo, Japan), at 0, 2, 6, and 24 h after the injection of FITC-conjugated *S. aureus* in mice under isoflurane anesthesia. All data on the increase in ear thickness were expressed as a percentage of the previous value in each individual mouse.

Macrophage culture assay

Cells of mouse macrophage cell line RAW264.7 (ATCC, Manassas, VA, USA) were grown in DMEM supplemented with 10% FBS, 4.5 g/L glucose, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mmol/L HEPES. Cells were seeded in 96-well culture plates at 5×10^3 cells/well, and cultured with the test compound (30 μmol/L) in the presence or absence of the suboptimal-dose 0.5 ng/mL mouse interferon-γ (IFN-γ) (PeproTech Inc., Rocky Hill, NJ, USA). After 3 days of incubation at 37 °C, culture fluids were removed and FITC-conjugated *S. aureus* at 30 μg/mL in warm medium was added. After 30-min incubation in a 5% CO_2 incubator, cells were harvested using cold PBS containing 2 mmol/L EDTA, then washed and treated for 15 min at 4 °C with phosphate buffer containing 4% paraformaldehyde (pH 7.4). FITC-positive cells were determined using a FACSaria II flow cytometer and DIVA 8.0.1 software. Activity was indicated as the MFI of whole cells.

Definition of group names and statistical analysis

A mouse not treated with *S. aureus* was defined as "normal", and a mouse treated with *S. aureus* alone (no KRT) was defined as a "control". The 0.25, 1, and 2 g/kg of KRT were described as KRT-L, KRT-M, and KRT-H, respectively. All values are expressed as mean ± SEM. Student's *t*-test was performed to compare normal and control groups. Tukey's, Tukey–Kramer, Steel's, Steel–Dwass, and Dunnett's tests were used to perform multiple comparisons between groups, or between control groups and drug-administered groups. A probability of <0.05 was considered significant.

Results

KRT reduces the number of living *S. aureus* in the cutaneous infection

We first investigated the effects of KRT on superficial skin infection model. The number of living bacteria in control mice was 726.3 ± 155.6 ($\times 10^5$ CFU/g) at 4 days post the inoculation (**Figure 1A**). KRT-M (1 g/kg) and KRT-H (2 g/kg) dramatically reduced the number of living bacteria to 6.2 ± 0.4 and 3.5 ± 1.1 ($\times 10^5$ CFU/g), respectively. Macroscopic lesion scores at 2 and 4 days were lower in mice treated with KRT-M or KRT-H than the control mice (**Figure 1B**). Representative macroscopic images are shown in **Figure 1C**. An extensive erosion with severe crust formation was observed in the back skin of control mice at day 2. In contrast, those of the mice treated with KRT-M and KRT-H showed milder skin lesion with less erosion and crust formation, and erosions and papules were hardly observed in KRT-H-treated mice at day 4. Histological observation revealed that KRT suppressed acanthosis and spongiosis in epidermis and edema in dermis (**Figure 1D**).The quantitative analysis of histological observations results are shown in **Supplementary Figure S1**.

KRT augments clearance of *S. aureus*

In order to focus on action of bacterial clearance, we next examined the effects of KRT in pseudo-infection model using FITC-conjugated dead *S. aureus*. We first examined, on whether KRT accelerates bacterial clearance, by measuring the amount of remaining FITC-conjugated *S. aureus* in the ear (**Figure 2A**). The amount of bacteria in

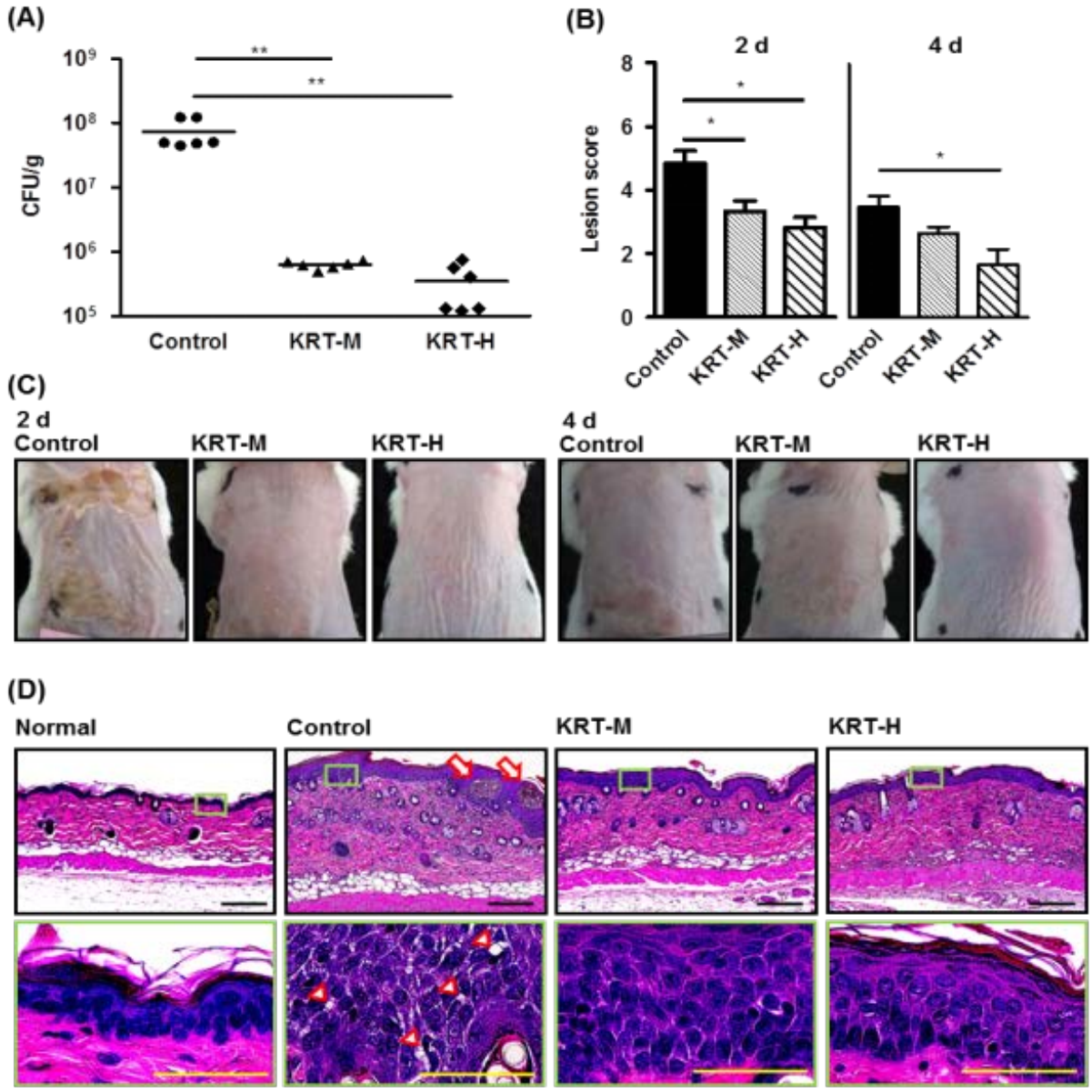

Figure 1. Keigairengyoto showed anti-microbial activities in a superficial skin infection model using living *Staphylococcus aureus*.
After tape stripping from the back skin of mice, *S. aureus* was inoculated at 1×10^7 CFU in 100 µL. Keigairengyoto (KRT, 1 and 2 g/kg) suspended in distilled water was administered orally to mice at 1 h before and 1, 2, and 3 days after the inoculation. The back skins were excised from mice on day 4. The number of living bacteria was measured on day 4 (**A**). Macroscopic damage scores of the infection sites were evaluated on day 2 and 4 (**B** and **C**). The skin sections on day 4 were stained with hematoxylin and eosin staining. Representative images are shown as low-power (upper panels) and high-power (lower panels); green boxes: the sites of each magnification; arrows: pustule; arrowheads: spongiosis; black bars: 200 µm; and yellow bars: 50 µm (**D**). $N = 2$ (normal) or 6 (control, KRT-M, and KRT-H). **: $P < 0.01$; *: $P < 0.05$ (Steel's test).

control mice was above 200 µg/ear at 2 h post-*S. aureus* injection and gradually decreased to less than half by 24 h. KRT-M significantly decreases the amount of bacteria at 6 and 24 h post-injection, though it shows no difference at 2 h. PDN exhibited no difference from the control at any time point. We histologically evaluated the bacterial clearance at 6 h post-injection in Gram-stained specimens. In control mice, gram-positive bacteria were deposited in the dermis of the ears (**Figure 2B**). KRT-M significantly decreased the deposition of gram-positive bacteria (**Figures 2B** and **2C**). In contrast, PDN exhibited no difference from control. These findings indicated that KRT promoted bacterial clearance.

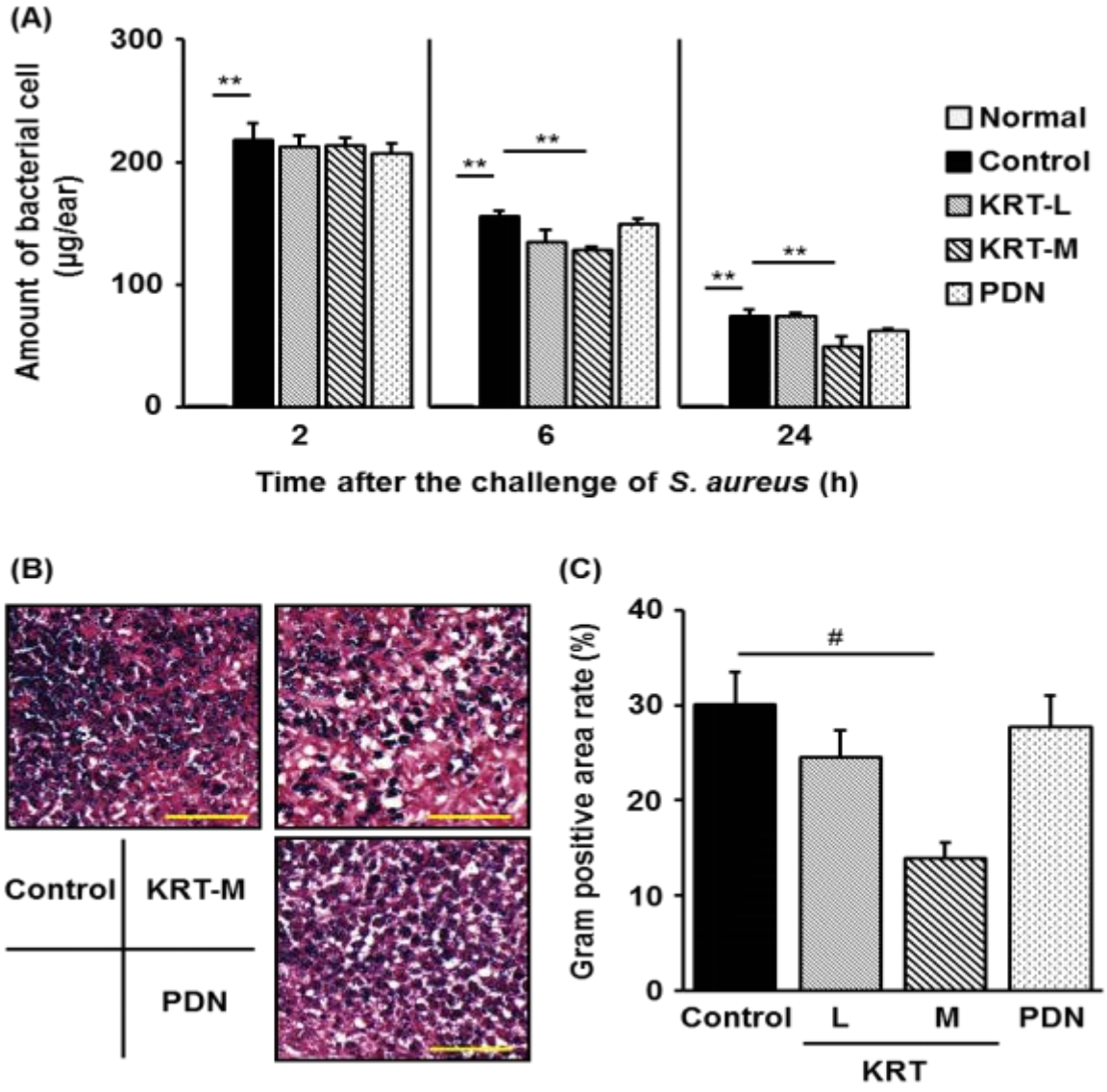

Figure 2. Enhancement of bacterial clearance by Keigairengyoto administration.
FITC-conjugated bioparticles of killed *S. aureus* were injected into both ears at 2×10^7 particles/10 μL/site. The ears were cut off at 2, 6, and 24 h after injection. Keigairengyoto (0.25 and 1 g/kg) suspended in distilled water or prednisolone (10 mg/kg) was administered orally to mice at 1 h before and 6 h after the injection. The amount of bacteria in the ears was measured biochemically at 2, 6, and 24 h after the injection (**A**). The ear sections were stained with Gram-Hacker's solution (**B**) and the deposition of Gram-positive bacteria in the lesion area was measured at 6 h after injection by an image-analysis technique (**C**). Yellow bars indicate 50 μm. $N = 8$. **: $P < 0.01$ (Tukey's test); #: $P < 0.05$ (Dunnett's test).

KRT reduces FITC-positive signals in clumps of *S. aureus*

Neutrophils were visualized as $Ly6G^+$ cells by fluorescent staining, and the localization of $Ly6G^+$ cells in clumps of FITC-conjugated bacteria was observed (**Figure 3**). In control mice, $Ly6G^+$ cells (red fluorescence) infiltrated mildly around the clumps of bacteria (green fluorescence) at 2 h, the cell infiltration had increased at 6 h, and an abscess had formed in the dermis with a decrease in FITC-conjugated bacteria at 24 h. In KRT-M-treated mice at 6 h, $Ly6G^+$ cells infiltrated more toward the center of bacteria clumps, which resulted in a pattern of dispersed FITC-positive signals at the periphery and compact highly FITC-positive signals in the center of the lesion. PDN-treated mice showed a similar distribution of $Ly6G^+$ cells in FITC-conjugated bacteria compared with control mice. Semi-quantitative image analysis of Ly6G-positive signals in the

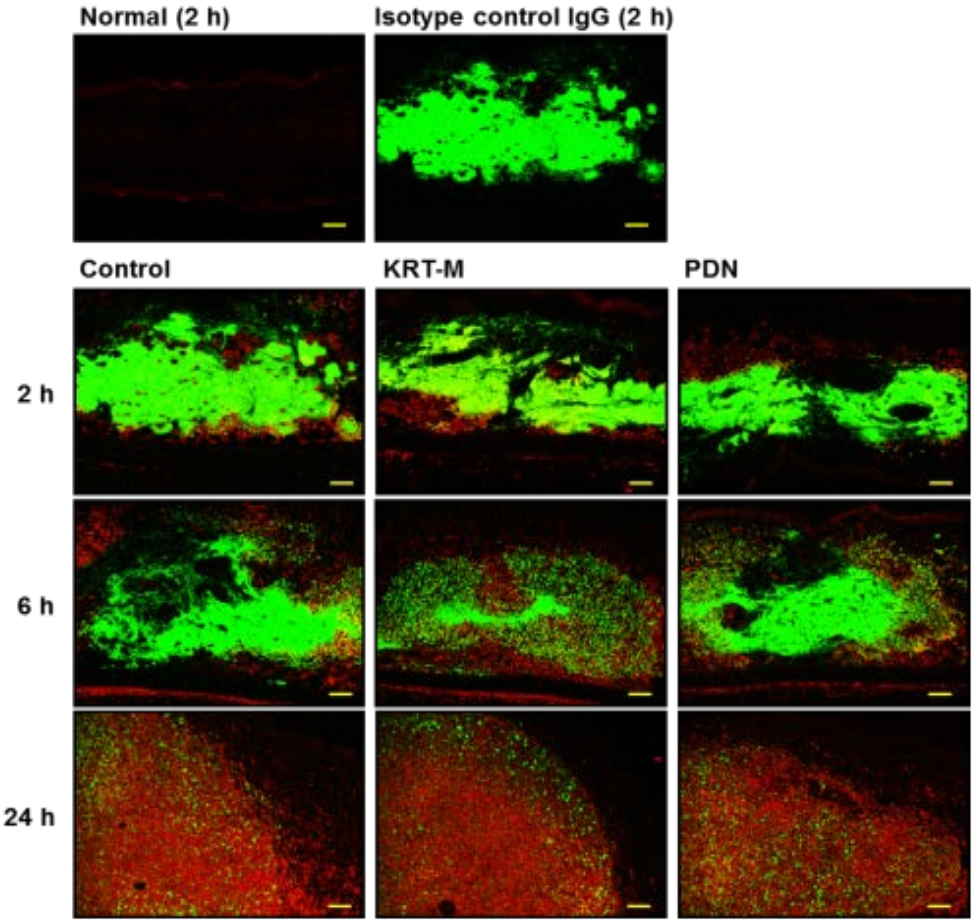

Figure 3. Keigairengyoto-induced enhancement of phagocytosis of Ly6G-positive cells.
The ears injected with FITC-conjugated *S. aureus* were cut off at 2, 6, and 24 h, and then subjected to immunofluorescence staining for Ly6G. Red and green indicate Ly6G-positive cells and FITC-conjugated *S. aureus*, respectively. Yellow bars indicate 50 μm.

lesions revealed no remarkable difference between groups at 6 h (data not shown). These results suggested that KRT treatment enhanced neutrophil penetration into the bacterial clumps at certain time periods, but did not significantly increase neutrophil numbers in the lesion.

KRT accelerates accumulation of macrophage-like cells around clump of *S. aureus*

HE-staining images at 6 h after the bacterial injection are shown in **Figure 4**. In normal mice without bacteria injection, resident macrophages—which had mononuclear morphology and larger cell size than resident lymphocytes—are observed. Macrophages-like cells, as well as polynucleated neutrophils, accumulated to the dermis around the bacteria clump in control mice at 6 h. In mice treated with KRT-M, the frequency of macrophages-like cells around the bacteria clumps was higher than control and showed larger abscesses at 6 h post-injection. Mice treated with PDN did not show a remarkable difference in macrophage-like cell infiltration compared with control mice.

KRT enhances F4/80$^+$ cell accumulation to the lesion

To clarify the cell-type of macrophage-like cells observed in **Figure 4**, fluorescent staining using anti-F4/80 antibody was performed. F4/80 protein is expressed mainly in resident macrophages, while monocytes also express it at a lower level than macrophages. F4/80$^+$-resident macrophages were observed in the dermis of normal mice, as shown in **Figure 5A**. F4/80$^+$ cells were accumulated around the bacteria clumps in control mice at 2 h after the bacterial injection. Mice treated with KRT-M showed more accumulation of F4/80$^+$ cells compared with the control, and this was confirmed by counting the number of F4/80$^+$ cells per unit area around the lesion site (**Figure 5B**). PDN-treated mice showed

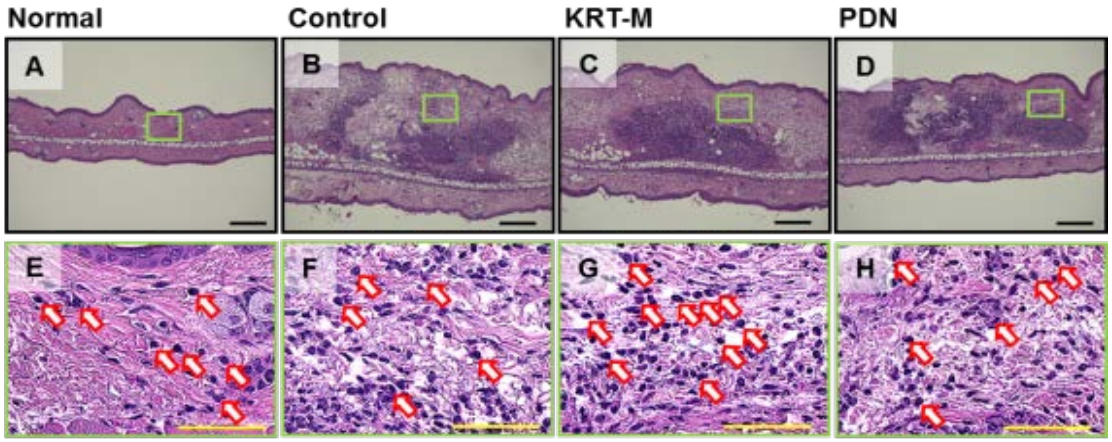

Figure 4. Histological analysis of cell kinetics by hematoxylin and eosin stain.
The ears were harvested at 6 h after the injection of FITC-conjugated *S. aureus* and evaluated histologically by hematoxylin and eosin staining. Representative images of low-power (upper panels) and high-power (lower panels) fields of the ear sections are shown for normal (**A** and **E**), control (**B** and **F**), KRT-H (**C** and **G**), and PDN (**D** and **H**). Green boxes indicate the sites of each magnification, and arrows indicate macrophage-like cells. Black and yellow bars indicate 200 and 50 μm, respectively.

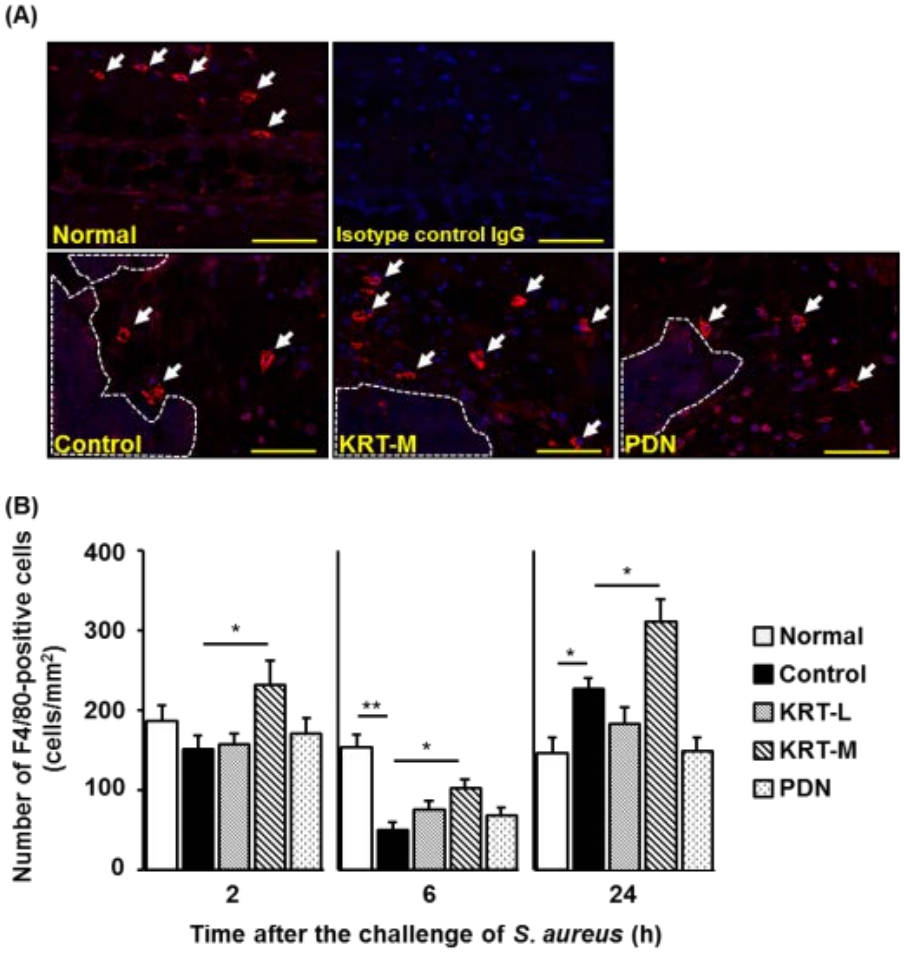

Figure 5. Keigairengyoto's effects on the accumulation of F4/80-positive cells at the lesion site.
(**A**) The ears injected with FITC-conjugated *S. aureus* were cut off 2, 6, and 24 h later and subjected to immunofluorescence staining for F4/80. Representative images of F4/80-positive cells are shown at 2 h after the injection. Arrows and areas surround by white dotted lines indicate F4/80-positive cells and clumps of bacteria, respectively. Yellow bars indicate 50 μm. (**B**) F4/80-positive cells were counted as the number of positive cells per unit area around the clump of bacteria or abscess in the ears at 2, 6, and 24 h after the injection. Yellow bars indicate 50 μm. $N = 7$ or 8. *: $P < 0.05$; **: $P < 0.01$ (Tukey-Kramer test).

levels of F4/80$^+$ cells similar to control mice at 2 and 6 h, but fewer F4/80$^+$ cells than control and KRT-M treated mice at 24 h. These results indicated that KRT-M increased the accumulation of F4/80$^+$ resident macrophages at the bacteria clumps.

Next, we investigated whether KRT affects the production of chemokines and cytokines related to macrophage functions. As KRT enhanced F4/80$^+$ cell accumulation compared to controls as early as 2 h, we examined the chemokine and cytokine production in the ears at 1 h after bacteria inoculation. KRT-M, however, showed no difference in the production of KC, MCP-1, and IL-1β at 1 h after the injection (**Figure 6**). We also measured them at 6 h post-injection and observed that KRT treatment did not alter the expression of these chemokines in the lesion (data not shown).

KRT augments phagocytosis by infiltrated cells *in vivo*

To quantitate numbers, populations, and phagocytic activity of macrophages and neutrophils in the lesions, FACS analysis was performed at 6 h after the *S. aureus* inoculation. In this study, CD11b$^+$Ly6G$^-$ and CD11b$^+$Ly6G$^+$ cells were designated as monocyte/macrophages and neutrophils, respectively. There was no change in the number of CD11b$^+$Ly6G$^-$ cells among the groups of normal, control, and KRT-treated mice. In contrast, the number of CD11b$^+$Ly6G$^+$ cells had clearly increased in the control group at 6 h after the injection compared to the normal group, but the difference between control and KRT-M-treated groups was not significant (**Table 1**). The number of FITC-positive CD11b$^+$Ly6G$^+$ cells, which were *S. aureus*-ingesting neutrophils, significantly increased in the KRT-M-treated group compared with the control group. Moreover, the MFIs of FITC in both CD11b$^+$Ly6G$^-$ and FITC-positive CD11b$^+$Ly6G$^-$ cells were significantly increased in the KRT-M-treated group. These findings indicated an adjuvant effect of KRT on the phagocytic response to a foreign pathogen. Representative blots are shown in **Supplementary Figure S2**.

KRT suppresses ear swelling due to *S. aureus* inoculation

It generally is thought that the activation of innate immune cells may exacerbate inflammatory symptoms. Therefore, we examined the effect of KRT on ear thickness (**Figure 7**). At 2, 6, and 24 h after the injection, the ear thickness of control mice had increased 1.7, 2.4, and 2.9 times compared with that of normal mice, respectively. We did not observe remarkable differences in ear swelling between control and KRT-M at 2 h after bacteria inoculation or abscess formation (**Supplementary Figure S3**). Of note, KRT-M significantly suppressed the

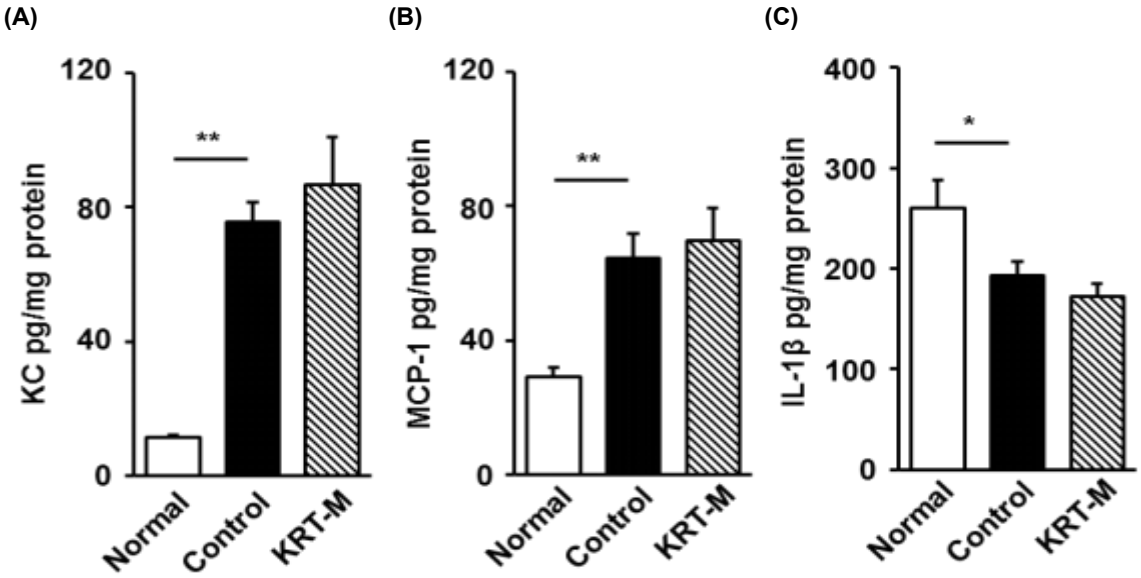

Figure 6. Keigairengyoto's effects on the production of chemokines and cytokines.
Keigairengyoto (KRT-M, 1.0 g/kg) suspended in distilled water was administrated orally to mice at 1 h before induction of dermatitis by intradermal injection of FITC-conjugated *S. aureus* into the ears at 2×10^7 particles/10 μL/site. The ears were cut off 1 h after the injection. KC (**A**), MCP-1 (**B**), or IL-1β (**C**) in homogenizations of the ears was quantified by their specific ELISA. $N = 7$ or 8. *: $P < 0.05$; **: $P < 0.01$ (Tukey-Kramer test).

Table 1. Cytometric analysis of phagocytes in ears of mice treated with Keigairengyoto

	Region	Cell number ($\times 10^4$ cells per ear)			MFI of FITC	
		Normal	Control	KRT	Control	KRT
Monocytes/macrophages						
$CD11b^+Ly6G^-$ (whole)	R1	27.2	29.0±2.8	28.8±1.8	113±16	190±29*
$CD11b^+Ly6G^-$ $FITC^+$	R3	-	5.8±0.9	7.4±1.1	484±39	673±55*
Neutrophils						
$CD11b^+Ly6G^+$ (whole)	R2	0.4	60.8±7.1	75.2±4.7	3,258±531	3702±374
$CD11b^+Ly6G^+$ $FITC^+$	R4	-	36.1±4.7	53.5±5.2*	5,247±589	5226±420

Keigairengyoto suspended in water was given orally to mice at a dose of 1 g/10 mL/kg, and 1 h later, FITC-conjugated *S. aureus* was injected to the ears. The ears were removed 6 h post-injection, and single cells were prepared by an enzyme-dispersing procedure. Phagocytic cells in the single ear cells were stained by anti-mouse Ly6G and anti-mouse CD11b and analyzed by flow cytometry. Data are shown as mean of 2 (Normal), 6 (Control), and 6 (KRT) ears per a group. Total numbers of harvested ear cells were 293, 322±18, 354±11 × 10^4, respectively. R1, R2, R3, and R4 indicate each region built in the dot plots of **Supplementary Figure S1**. There was no difference among groups. *: $P < 0.05$ significant with the Student's *t*-test.

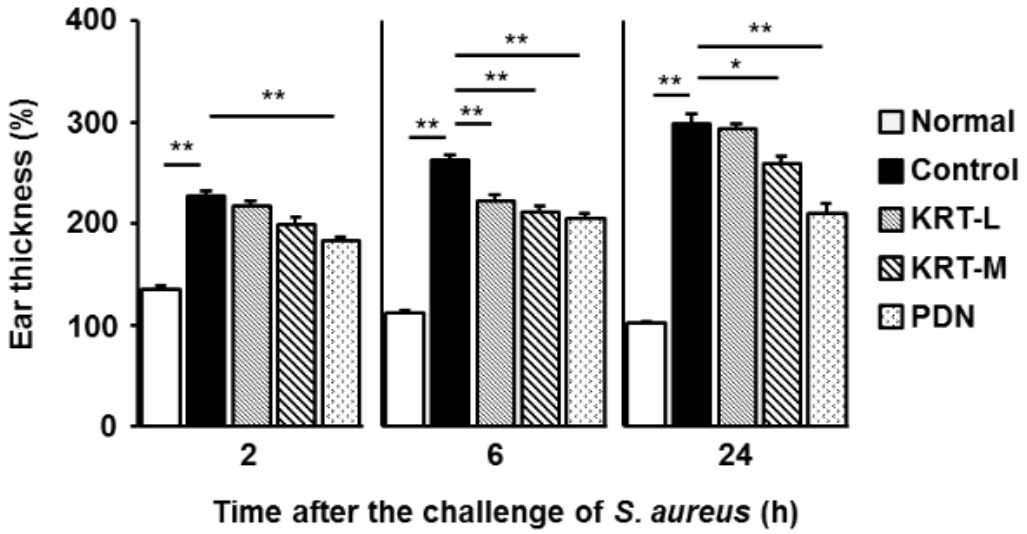

Figure 7. Suppression of ear thickness by Keigairengyoto administration.
Dermatitis was induced by intradermal injection of FITC-conjugated *S. aureus* into the ears. Ear thickness was measured at 0, 2, 6, and 24 h after injection. $N = 8$.*: $P < 0.05$; **: $P < 0.01$ (Steel-Dwass test).

increase in ear thickness at 6 h, though mice treated with KRT-M showed larger abscesses at 6 h post-injection, as shown in **Figure 4**. At 24 h, mice treated with KRT-M showed significantly thinner ears and smaller abscesses than control mice (**Supplementary Figure S3**). PDN also significantly suppressed ear swelling as well as abscess formation by *S. aureus* inoculation at all time points.

Active compounds of KRT augment phagocytosis by macrophages *in vitro*

Finally, we examined whether absorbed compounds related to KRT can augment macrophage functions *in vitro*. As shown in **Table 2**, genistein 7-*O*-glucuronide remarkably enhanced phagocytic activity in the macrophage cell line RAW264.7. Liquiritigenin 7-*O*-glucuronide also promoted phagocytosis by RAW264.7, while liquiritigenin 4'-*O*-glucuronide, hesperetin 7-*O*-glucuronide, 18β-glycyrrhetinic acid, and cimifugin were inactive in the present test.

Discussion

In this study, we demonstrated that KRT decreased the number of living bacteria and suppressed the development of the bacteria-induced skin lesions (**Figure 1**). Furthermore, we observed in the pseudo-infection model that KRT significantly decreased the numbers of dead bacteria at 6 and 24 h post-injection by measuring FTTC-signals in the homogenizations of the inflamed ears (**Figure 2A**). As a complementary test, the histological examination by Gram-

Table 2. Effects of Keigairengyoto-related compounds on macrophage phagocytosis

Test Sample	IFN-γ	Phagocytosis (MFI)
No IFN-γ	-	116±1
Control	+	134±2
Genistein 7-*O*-glucuronide	+	457±22**
Liquiritigenin 7-*O*-glucuronide	+	181±12*
Liquiritigenin 4'-*O*-glucuronide	+	139±3
Hesperetin 7-*O*-glucuronide	+	127±1
18β-glycyrrhetinic acid	+	143±4
Cimifugin	+	135±2

Mouse macrophage-like RAW264.7 cells were seeded in 96-well culture plates at 5×10^3 cells/well and cultured with test samples (30 μmol/L) in the presence or absence of suboptimal dose of 0.5 ng/mL mouse interferon-γ (IFN-γ). The culture fluids were removed after 3 days of culture and replaced with fresh medium containing FITC-conjugated *S. aureus* (30 μg/mL), followed by an additional culture for 0.5 h. The cells were harvested, and the FITC-positive cells were measured by FACSaria II. $N = 3$. *, **: $P < 0.05$, 0.01 *vs.* IFN-γ alone control (Dunnett's test), respectively.

stain indicated that KRT reduced *S. aureus* per unit lesion area 6 h after the injection (**Figure 2B**). Taken together, it is plausible to conclude that KRT enhances *S. aureus* clearance from infectious sites.

KRT is reported to have an anti-bacterial activity against *S. aureus* and *Propionibacterium acnes* in *in vitro* assays[22,23]. Therefore, the result of KRT in the infection model using living *S. aureus* may be due to direct attack against to the *S. aureus*. On the other hand, pseudo-infection experiment was performed in order to eliminate a possibility of direct bactericidal effect of KRT. It is plausible to conclude that KRT enhances the potentials of phagocytic cells, such as macrophage and neutrophils, and promotes bacterial clearance. It is unclear how much the enhancing effect of KRT on innate immune cells contributes to reducing the number of living *S. aureus* in the infection model. However, the enhancing effect of KRT is surely involved at least partially in bacterial clearance in the present infection model. One of the important strategies is to investigate a mechanism by which the bioactive flavonoids shown in **Table 2** enhance macrophage functions. We should examine cell-based kinetic study focusing on innate immune cells in the superficial skin infection model to clarify the effect of KRT, as well as the bioactive flavonoids, in more detail.

The histological examinations by HE and immunostaining using anti-F4/80 antibody indicated that KRT augmented resident macrophages accumulation around the clumps of bacteria or lesion site. Interestingly, KRT did not increase the number of $CD11b^+Ly6G^-$ cells in FACS analysis, which represented the number of macrophages per ear at 6 h post-injection (**Table 1**). Expression of F4/80 is heterogeneous and is modulated during macrophage maturation and activation. Monocytes that circulate in the bloodstream also express F4/80 on the surface, but the level is lower than that on resident macrophages. Langerhans cells and a subpopulation of dendritic cells also express F4/80. Since Langerhans cells reside mostly in the epidermis, dermal $F4/80^+$ cells measured in our study were mainly resident macrophages but not Langerhans cells. As the dermal resident macrophages are high in $F4/80^+$ and both resident macrophages and circulating macrophages are $CD11b^+Ly6G^-$, the accumulation of $F4/80^+$ macrophages around the bacteria clump is supposed to depend on the migration of resident macrophages from the nearby skin but not from the systemic circulation. Moreover, KRT increased phagocytosis by macrophages, which was shown as a higher MFI of FITC conjugated to *S. aureus* in $CD11b^+Ly6G^-$ cells than that of control group at 6 h in FACS analysis. FACS analysis performed at 2 h post-injection as well as the 6 h protocols similarly showed that KRT enhanced macrophage functions (data not shown).

Interestingly, our study found that the numbers of $F4/80^+$ cells per area around the lesion site in control groups decreased transiently at 6 h after the bacterial injection and then increased at 24 h, compared with normal groups. It is reported that *S. aureus* infection induces pyroptosis of resident macrophages, which is a pro-inflammatory programed cell death[24]. Macrophages that died by pyroptosis release alarmins including IL-1β, which induces recruitment and activation of neutrophils[25]. Cell death by pyroptosis is considered to play a crucial role for efficient elimination of bacteria. In an animal model of *S. aureus* skin infection, $F4/80^{high}$ dermal macrophages initially decrease post-infection and expand later due to renewal by incoming $Ly6C^{high}$ inflammatory monocytes[7]. Thus, our finding of the transient decrease of $F4/80^+$ cells may relate to the

characteristic modulation of F4/80-expressing cells during macrophage maturation and activation.

Neutrophils, represented as $CD11b^+Ly6G^+$ cells by FACS analysis, increased after inoculation with FITC-conjugated dead *S. aureus* (**Supplementary Figure S2**). Although KRT treatment did not increase the number of $CD11b^+Ly6G^+$ cells in the whole ears compared to the control, FITC-positive $CD11b^+Ly6G^+$ cells were significantly more numerous in KRT-treated mice than in the control (**Table 1**). The MFI of FITC in $CD11b^+Ly6G^+$ cells, which is a parameter of phagocytic potential per cell, was much higher than that in $CD11b^+Ly6G^-$ cells. Accordingly, $Ly6G^+$ cells were observed more frequently in the clump of FITC-positive *S. aureus* in the KRT-treated group at 6 h post-injection, and the histological images of FITC-positive *S. aureus* clumps were evidently fewer in KRT-treated group than that of control group (**Figure 3**). Circulating neutrophils are recruited rapidly to infectious areas through chemotactic signals and eliminate bacteria by bactericidal and phagocytic mechanisms[2,3,26]. Depletion of neutrophils delays bacterial clearance and causes lethality in mouse lung infection[27]. Considering that neutrophils are the most professional effect or cells against foreign pathogens, the capacity of KRT to promote bactericidal functions of neutrophils should be noted.

Some *kampo* medicines including KRT are flavonoid-rich. To evaluate the effects of KRT on phagocytosis by macrophage, we investigated the absorbed flavonoid compounds, which would be detected in the blood of mice that were given KRT orally. We recently demonstrated that jumihaidokuto, another *kampo* medicine that contains the same flavonoid-rich components as KRT, suppresses *Propionibacterium acne*-induced dermatitis by modulating macrophage function[19]. Considering our pharmacological and blood pharmacokinetic studies of jumihaidokuto[18,19], genistein 7-*O*-glucuronide, liquiritigenin 7-*O*-glucuronide, liquiritigenin 4'-*O*-glucuronide, hesperetin 7-*O*-glucuronide, 18β-glycyrrhetinic acid, and cimifugin were chosen for *in vitro* assays of macrophage phagocytosis in this study. Among these compounds, genistein 7-*O*-glucuronide was the most active in enhancement of phagocytosis. We recently clarified that glucuronides of phytoestrogen flavonoids, such as genistein and liquiritigenin, enhance macrophage functions *via* de-conjugation and stimulation of nuclear estrogen receptor in macrophages, resulting in the up-regulation of phagocytosis and expressions of complement receptors, Fc-receptors, chemotactic receptor, and so on[28]. Flavonoids of KRT would work directly on the innate immune system *in vivo*.

Proinflammatory cytokines and toxic radicals produced by activated macrophages would induce inflammatory symptoms such as edema and reddening of the skin. We measured ear thickness as a general clinical marker of skin inflammation after dead bacteria inoculation. To our surprise, although KRT recruited more macrophages and neutrophils to the bacteria clumps, KRT rather suppressed ear thickness at 6 and 24 h post-injection compared to the control. Histological observation in the infection model of living bacteria revealed that KRT suppressed edema of the epidermis and dermis in the lesion skin (**Figure 1D**). We observed that skin lesions of the KRT-treated group did not show obvious differences in KC, MCP-1 and IL-1β expression compared to the control group at 1 h post-injection (**Figure 6**). IL-1β was also measured at 6 h after the injection, but no difference was observed in KRT-treated mice (data not shown). These indicated that inhibitory effect of KRT on ear thickness did not depend on the modulation of proinflammatory cytokines induction.

KRT-treated mice showed thinner ears at 6 h while the bacteria had not been completely phagocytized and cleared. KRT-treated mice also showed less ear thickness and clearly smaller abscesses than the control at 24 h, suggesting that KRT enhanced the bacteria clearance at this point. PDN, which has strong inhibitory effects on various inflammatory responses, also suppressed ear thickness without affecting bacteria clearance. Therefore, KRT may have mechanisms other than promoting macrophage functions to suppress skin inflammation. In bacteria-infected sites, damaged cells, dead cells, and phagocytes produce toxic radicals. An excessive production of toxic radicals in the extracellular space can damage the surrounding healthy tissues and cause secondary non-infectious inflammation[29,30]. KRT has many blood-absorbing anti-oxidant constituents[31,32]. Our previous study demonstrated that liquiritigenin 7-*O*-glucuronide emerged in blood immediately after oral administration and exerted anti-oxidant activity[18]. Blood pharmacokinetic studies of active compounds originating from KRT could uncover the detailed mechanisms by which KRT exerts anti-inflammatory functions.

Conclusion

This is the first report to confirm that oral administration of KRT promoted bacteria clearance in mice. KRT activated resident macrophages to migrate to the bacteria lumps and promoted

phagocytosis against FITC-conjugated *S. aureus*. *In vitro* assays showed that absorbed flavonoids such as genistein 7-*O*-glucuronide remarkably promoted phagocytic activity in a macrophage cell line. Moreover, KRT enhanced the number of FITC-positive neutrophils in the ears, probably through the activation of resident macrophages (**Figure 8**). Thus, KRT activated the innate immune system, which was supported by the results in the infectious and pathophysiological model of the living bacteria.

Authors' contributions

J Koseki and A Kaneko performed experiments, analyzed data, prepared figures and tables, interpreted results of experiments, and drafted the manuscript. Y Matsubara, K Sekiguchi, and S Ebihara performed experiments and analyzed data. S Aiba interpreted results of experiments. K Yamasaki interpreted results of experiments and revised the manuscript. All authors reviewed and edited the manuscript, and approved the final version of the manuscript.

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Appendix

The present manuscript contains three supplementary figures.

Conflict of interest

Dr. Kenshi Yamasaki and Dr. Setsuya Aiba received research grant support from Tsumura & Co. Junichi Koseki, Atsushi Kaneko, Kyoji Sekiguchi, Yosuke Matsubara, and Satomi Ebihara are employed by Tsumura & Co.

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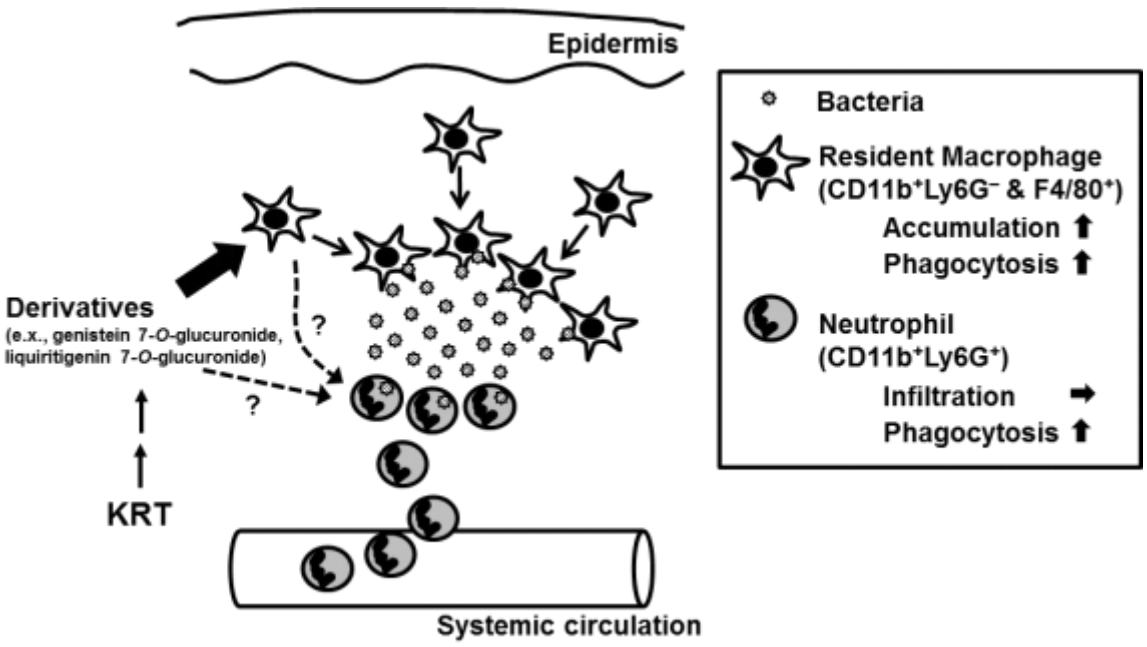

Figure 8. Schema of possible action of KRT on bacterial clearance.
KRT enhances the functions of innate immune cells to accelerate the accumulation of resident macrophages ($CD11b^{+}Ly6G^{-}$ and $F4/80^{+}$) around bacteria clumps and bacterial phagocytosis by macrophages and neutrophils ($CD11b^{+}Ly6G^{+}$). KRT derivatives, such as genistein 7-*O*-glucuronide and liquiritigenin 7-*O*-glucuronide, may activate resident macrophages directly and augment host innate immune functions.

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REVIEW ARTICLE

Dynamic relationships among tumor, immune response, and microbiota

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ABSTRACT

Recently, the analysis of microbiota has been of interest not only for the clarification of the molecular mechanisms of disease etiology, but also the discovery of novel strategies for treatment. Following the development of "next-generation" sequencing, novel areas have been discovered in microbiota; however, in oncology, the relationships between microbiota and cancer have not been fully clarified. In recent literature, surprisingly, detection of gut microbiota in tumor issue itself has been reported. Microbiota might play an important role in carcinogenesis. However, this phenomenon is not well understood, and research in this area has just begun. In the past five years, a paradigm shift has occurred in cancer treatment due to immunotherapy. Immunotherapy has made cure possible even in advanced cancer patients with not only melanoma but also non-small cell lung cancer and others. In this review, we discuss the mechanisms of novel immunotherapies, checkpoint inhibitors, and the relationship between microbiota and immunotherapy. It is of significance to clarify this relationship because it may lead to the discovery of predictive markers for immunotherapy and promote clinical efficacy. Finally, we also mention our activities in the construction of a big database for information on immunotherapy and microbiota, which may lead to excellent possibilities of discovering novel strategies for more effective cancer treatments, and may accelerate the alteration of cancers to the classification of chronic nonfatal disease.

Keywords: *gut microbiota; immunotherapy; checkpoint inhibitors; cancer treatment; immunoresponse*

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Introduction

In this review, an overview of the relationships of gut microbiota among tumor, immunological response, and therapeutic effect is presented. Gut microbiota are mainly located in the human gastrointestinal tract, and the microbes number in the hundreds of trillions in healthy subjects[1,2]. It has been reported that thousands of kinds of gut microbiota are in the body, which is a few times more than the kinds of adult human cells[3]. Before the advent of next-generation sequencing methods (NGS), our understanding of gut microbiota was not clear, due to technological limitations. For example, *Escherichia* (*E.*) *coli* had seemed to be the most common bacteria in gut because of its high frequency of detection by the previous methods. However, after NGS, *E. coli* has been found to be not so common in the human gut—anaerobic bacteria are more frequent. It is said that microbes become established in the gastrointestinal tract until the host reaches three years of age, and then the microbes reach an adult state as gut microbiota[4]. Gut microbiota and human beings are co-evolutionary, in a win-win symbiosis[5–7]. Recently, there have been many studies and much research on the relationships between the gut microbiota and human disease[8–11]. Gut microbiota seem to be strongly correlated with various kinds of disease, such as inflammatory bowel disease (IBD)[12–15], diabetes mellitus (DM)[16–18], central nervous disorder (CND)[19–24], allergic diseases[25–29], and infectious diseases[30]. On the other hand,

the relationship between tumor and microbiota is not well clarified, especially from the viewpoint of immunology. Here, we focus on the relationship between gut microbiota and cancer, which recently has been noted. Interestingly, the efficacy of a checkpoint inhibitor as tumor immunotherapy has also been reported to be related with the host gut microbiota[31–33].

Detection of microbiota

In general, most published analyses of gut microbiota have used a statistical analysis system (SAS). If the homology of the genetic information of microbes in different tests is over 96%, the species are defined to be the same because of the error in polymerase chain reaction (PCR). Before the appearance of the next-generation sequencer, many genetic analysis reports were made and the results were pooled into a database. In the genome of a bacterium composed of several million base pairs, there is a highly polymorphic region of approximately 1,500 base pairs, the "16S ribosomal RNA region." In the 16S region, there are nine hypervariable regions consisting of several hundred base pairs, with a characteristic arrangement depending on the kind of bacteria. Also, it is known that this hypervariable region is conserved within a bacterial species, and therefore, to identify a kind of species, it is good enough to sequence the 16S region.

Hattori *et al.* have reported that bacterial deoxyribonucleic acid (DNA) encoded in V1 and V2 region of 16S ribosomal ribonucleic acid (rRNA), which contains about 300 genes, is sufficient as the target for determination of the species of a microbe[34,35] because of the high species-specificity of the 16S rRNA. It is technically of importance to break the hard cell wall of bacteria in the extraction of DNA from samples. After creating a library based on PCR data using specific primers we had available, we used a PGM system (Thermo Fisher Scientific K.K., Yokohama, Japan) to read the sequences from the library. There are several other methods for determining gut microbiota as well; however, so far, there is insufficient data to evaluate these methods, and further studies or experiments are needed to clarify them. Special care must still be taken when determining the bacterial species until the detection methods are appropriately evaluated.

Tumors and microbiota

Interestingly, our group and others have successfully detected gut microbiota in tumor itself, even in tumors derived from outside of the gastrointestinal tract. Cluster analysis methods have demonstrated totally different patterns in comparison between microbiota in tumor tissue and the adjacent normal tissue, clearly indicating that bacteria in gut move from gut or elsewhere in the body to the tumor via blood or lymphatic vessels, and the kinds of bacteria in cancer tissues are different from those in normal tissues. The biology of tumor microbiota is not yet fully clarified. Below, we discuss some types of cancer from the viewpoint of microbiota.

Breast cancer

Although the molecular biological etiology of breast cancer is not yet fully known, it has been reported to involve a combination of genetic and environmental elements. Along with genetics, environmental factors contribute to breast cancer development, but what these exact environmental factors remain unknown. Although results of their analysis were different from ours, some interesting studies may offer support for certain environmental factors being associated with an increased incidence of breast cancer[36,37]. Urbaniak *et al*. demonstrated that breast tissue contains a diverse population of bacteria[38,39]. Using the analysis of 16S rRNA, they showed that the pattern of microbiome is completely different between the adjacent normal breast tissue from women with breast cancer and breast tissue from healthy controls. Furthermore, they also observed that the pattern of bacteria is almost similar between adjacent normal breast tissue and breast tissue sampled directly from breast cancer. This might indicate that the development of breast cancer is affected by patients' microbiome as one of the environmental elements. In their publications, patients with breast cancer had higher relative abundances of *Bacillus*, *Enterobacteriaceae,* and *Staphylococcus* in comparison with those in healthy controls. These bacteria species induce DNA damage, possibly double-stranded DNA breaks. Bacteria that have a function of causing DNA damage were detected in breast cancer patients; on the other hand, there were lower levels of some lactic acid bacteria, known for their beneficial health effects, including anticarcinogenic properties. *Bacillus* is more frequently detected in breast cancer patients compared with healthy controls. Although *Bacillus* does not induce DNA damage as do *E. coli* and *S. epidermidis*, it may have other carcinogenic effects, such as metabolization of hormone and/or stimulation of cell proliferation. On the other hand, in a role of prevention, *Lactococcus* and *Streptococcus*, which are higher in healthy women than in breast cancer patients, may show anticarcinogenic properties. However, it is very difficult to conclude that, in terms of mechanism, some bacteria by themselves cause a high incidence of breast carcinoma because patients

have the prevention pathway of immunosurveillance, which will be discussed later.

Colorectal cancer

Recent reports have clearly demonstrated that *Fusobacterium* (*F.*) *nucleatum* is one of the major risk factors for colorectal cancer[40–42]. Some studies suggested that *F. nucleatum* showed immunosuppressive activities of T cell response in the tumor microenvironment. This indicated that microbiome and immunoresponse are strongly associated with carcinogenesis. Nosho *et al.* and Mima *et al.* demonstrated that *F. nucleatum* in colorectal cancer activates and proliferates myeloid-derived immune cells, typically myeloid-derived dendritic cells (DCs) and M2 macrophages, which strongly induce immunosuppressive action of T cells in the tumor microenvironment[43,44]. These cells produce a number of reactive oxygen species (ROS) and inflammatory cytokines, such as Interleukin (IL)-10. There may be some possibility that *F. nucleatum* stimulates and produces microRNA-21, which also stimulates the production of IL-10 and prostaglandin E2. These products strongly suppress antitumor T cell-mediated adaptive immunity via regulatory T cell in the tumor microenvironment. ROS also causes epigenetic silencing of the mismatch repair protein MLH1 to induce microsatellite instability. The mechanisms regarding the association between immune cells and molecular alterations caused by *F. nucleatum* have not been well clarified. However, it is clear that gut microbiota in tumor affect the host's immune response in the tumor microenvironment.

Gastric cancer

Helicobacter (*H.*) *pylori* selectively colonize the gastric epithelium, and are believed to be a major player in the etiology of gastric cancer. *H. pylori* infection begins in childhood and persists for the whole life of the host. In Japan, it is common to have *H. pylori,* which is thought to be a main reason that gastric cancer is one of the major cancers in Japan. Between approximately 1% and 3% of *H. pylori*-infected persons suffer gastric adenocarcinoma.

Tumor immunotherapy and microbiota

Tumor immunology and immunotherapy

Many recent publications have clearly demonstrated that microbiota are among the key elements for determination of antitumor effects. The efficacies of tumor immunotherapy of programmed death (PD) 1 and PD-ligand (L) 1 and cytotoxic T lymphocyte antigen (CTLA)-4 antagonist, known as immune checkpoint inhibitors, are determined by genetic and environmental factors. Microbiota may affect the drug efficacy, abrogation of anticancer effects, and mediation of toxicity of chemotherapeutic drugs[45–49]. This has not yet been completely clarified from the viewpoint of molecular biology, but some metabolites from microbiota may significantly affect these phenomena. However, it is difficult to explain how only a few kinds of microbiome could determine the phenomenon and antitumor activity *in vivo*.

Interesting studies in this regard have been published recently. Antitumor effect of immunomodulatory drugs, anti-CTLA-4 antibody, and anti-PD-L1 antibody, has been reported to strongly correlate with the gut microbiota in a murine model[31,32]. Recently, tumor immunotherapy using these types of drugs has demonstrated extremely strong antitumor effect in the clinical setting. Many clinical trials have revealed significant effect, not only in progression-free survival, but also in overall survival.

In terms of T cell activation, the first signal is the binding with major histocompatibility complex (MHC)-peptide complex and T cell receptor (TCR). After antigen-presenting cells (APC), such as DCs or macrophages, present tumor antigens onto their cell surface as the complex of MHC-tumor-derived peptide, the T cell, as a first signal, recognizes this complex by the TCR. Cluster differentiation (CD)28 on the T cell is a costimulatory molecule on activated T cell binding to B7.1 molecule as a second signal of the activated T cells. The third signal of the activated T cell is a cytokine release, such as IL-2, for maintenance to activate T cell. To continue the activation of the T cell, CD28 on the T cell binds to B7.1 molecule, and then cytokine is released from the activated T cell to continue proliferation and activation of T cells as a third signal. CTLA-4 is important for the inactivation of T cells[50–52]. To shut down the activation of T cells, CTLA-4 is up-regulated to the activated T cell surface. CTLA-4 has 100-times higher binding affinity to B7.1, and therefore CD28 does not bind to B7.1. This induces the activated T cell to be inactive, and therefore the T cell tones down to form a kind of resistance. Anti-CTLA-4 antibody strongly blocks the binding CTLA-4 molecule and B7.1 molecule, enabling the T cells to continue to activate and produce the lymphocyte-stimulatory cytokines. *In vivo*, anti-CTLA-4 antibody is thought to be involved at the lymph nodes for the activation of T cells. It has been known that CTLA-4 molecule is also highly expressed to regulate T cells and block the activation of regulatory T cells, inducing the continuation of the T cell activation.

Anti-CTLA-4 antibodies, ipilimumab and tremelimumab, showed strong antitumor effect and revealed excellent clinical efficacy against melanoma. An important characteristic of anti-CTLA-4 antibodies is the duration of their antitumor effect [53,54]. The overall survival of 4,846 melanoma patients treated with ipilimumab was statistically analyzed using Kaplan-Meier methods[53]. Surprisingly, almost 20% of patients who were treated with ipilimumab showed prolonged survival: patients who were expected to live for 3 years have lived for 10 years. This response of overall survival is said to form a "kangaroo-tail phenomenon" on the Kaplan-Meier curve[55].

On the other hand, in the periphery of tumor site, the axis of PD-1 and PD-L1 is important for T cell peripheral dysfunction[56–59]. In contrast to CTLA-4 blockade, PD-1 binds to PD-L1, which is expressed on the tumor and/or immune suppressor cells in the tumor microenvironment. The activated T cells express PD-1, these T cells bind to its ligand PD-L1, and then the activated T cells become inactive. As a result, the T cells neither proliferate nor kill the tumor.

PD-1 was discovered by Ishida *et al*. in 1992 [60]. At first, PD-1 was thought to be one of the programmed death factors. In a genetically modified murine model, CTLA-4 knock-out is fatal at very young age or as a fetus, whereas PD-1 knock-out mouse is healthy until some weeks of age. The activated T cell highly expresses PD-1 continuously. On the other hand, PD-L1 is known to show two types of expression pattern. One type is constitutive, as some types of tumor highly express PD-L1 as a characteristic. The other is an inducible pattern. The activated T cell produces IFN-γ at the recognition of tumor. IFN-γ induces the expression of PD-L1 on the tumor and on the cells in the tumor microenvironment via nuclear factor-kappa B (NF-κB) or interferon regulatory transcription factor (IRF)-1[61]. This may be one reason why PD-L1 is not a good biomarker for anti-PD-1/PD-L1 therapy, because the IFN-γ release and/or PD-L1 expression is not very constant in the tumor microenvironment in terms of timing and quantity.

Anti-PD-1 antibody and anti-PD-L1 antibody are registered by the US Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) against several kinds of tumor, including melanoma, non-small cell lung cancer (NSCLC), and so on. One anti-PD-1 antibody, pembrolizumab, showed strong antitumor effect and clinical efficacy[62,63]. It was demonstrated that pembrolizumab shows significant clinical benefit, namely improvement of overall survival (OS), compared with platinum doublet as a first-line chemotherapy against NSCLC. In advanced NSCLC, platinum doublet has been a first-line chemotherapy for 30 years; however, in this clinical trial, Keynote-024, OS is significantly longer with pembrolizumab than with platinum doublet. This clearly indicates that the immunological response against tumor cells in our body has a much stronger antitumor effect than chemotherapy when the breaks on the immune checkpoints are released.

Mechanism of tumor immunology in humans

The following clinical evidence has provided answers to several previous fundamental questions regarding tumor immunology in humans: First, immunological reactions that recognize and kill tumor do not exist or do not function in (advanced) cancer patients. If we have an immunological response against tumor, we do not suffer from cancer. It has been clarified that humans have immunological response against cancer, even in advanced stages. Due to some clinical trials of anti-PD-1 antibody immediately showing antitumor effect, it clearly demonstrates that antitumoral immunological response is already in the tumor microenvironment; otherwise, it would take time to newly establish acquired types of immunological response against tumor. Secondly, even if we have immunological response against cancer, it might not be very strong. For example, if antitumoral immunological response is observed in only a few cases out of 100, it is not sufficient in the clinical setting. This indicates that only a very small number of patients have a benefit from their own immunological response against cancer. Recent early clinical trials have indicated that response rate is significantly high even in advanced cancer, and demonstrated that a meaningful number of patients are possible candidates to benefit from their own immunological response to tumor. Lastly, for each type of cancer, some significant drugs have been registered and they show clinical benefit even if it is not very strong. Until a few years ago, most believed that drugs utilizing the immunological response against cancer might not be effective because immune reactions are not strong enough to show clinical efficacy comparable to the presently registered anticancer drugs. Recent clinical trials have demonstrated that the drugs that utilize immunological response against cancer are so potent as to show higher antitumor effect compared with previously existing antitumor treatment such as chemotherapy.

Predictive biomarkers for immunotherapy and microbiota

As shown in these clinical results, immunotherapy has demonstrated clinical efficacy for cancer patients. However, it has not for all cancer patients, and it is limited. In the Keynote-024 study[63], it was shown that only patients with >50% of PD-L1 expression benefit from clinical efficacy. The big questions raised are which patients will show clinical benefit, and what are the markers for prediction? Basically, immunotherapy is much different from chemotherapy and radiotherapy (**Figure 1**). Chemotherapy and radiotherapy already show antitumor effect before administration, as when anticancer drugs are co-cultured with tumor cells or tumor cells are bombarded with X-rays *in vitro*. On the other hand, immunotherapy itself does not show antitumor activity before administration, as it is activated in the patient's body after administration. The immune checkpoint inhibitor does not show antitumor effect *in vitro*. In other words, in immunotherapy, patients make immunological drugs become active in their own body. It is crucial to understand how to make immunotherapy more effective, such as with combination therapy and discovery of predictive biomarkers.

Chemotherapy and radiotherapy themselves are already active before treatment; however, immunotherapy is not active before administration. *In vivo*, our body makes immunological drugs active (to show antitumor effect), and therefore it is necessary to understand the mechanisms in terms of environmental elements such as microbiota.

It is believed that the efficacy of an anticancer drug is derived from genetics, the drug itself, and host factors. Immunological drugs seem to be strongly dependent on host factors. Recently, some literature had demonstrated that tumor-infiltrating lymphocytes are strongly correlated with the clinical efficacy of checkpoint inhibitors[64]. The amount of CD8-positive lymphocytes in tumor microenvironment is strongly correlated with the decrease in size of tumor after checkpoint inhibitor therapy. In theory, this phenomenon is understood clearly. CD8-positive cytotoxic T lymphocytes are recognized as elements from tumor cells, which recently brought focus on neoantigens, and are clonally expanded and would kill tumor cells. However, activated T cells also express PD-1, and tumor cells themselves or the tumor microenvironmental immune cells express PD-L1, which induces tolerance of activated T cells. After the shutting down of the PD-1 and PD-L1 pathway using anti-PD-1 antibody, T cells re-activate and kill tumor cells, and demonstrate strong antitumor activity.

A core question is raised as to which patients show CD8-positive T cell in tumor tissue, supposedly a responder for immunotherapy. This question is important for the discovery of predictive markers for immunotherapy. Recently, to answer this question, the relationships between immunotherapy and microbiota were focused on (**Table 1**). Two murine studies were published[31,32]. The French group[31] demonstrated that antitumor effect using anti-CTLA-4 antibody is strongly related to *B. fragilis* and *B. thetaiotaomicron*. At first, it is an interesting observation that specific pathogen-free (SPF) mice showed potent antitumor effect, while germ-free mice did not show significant antitumor effect. SPF mice still had microbiota in them. The group hypothesized that some kinds of microbiota affect the antitumor effects. As their conclusion, they found that *B. fragilis* and *B. thetaiotaomicron* are the key bacteria to show the antitumor effect. They thought that the mechanism for this phenomenon is that these bacteria stimulate DCs, and DCs activate immune response, including CD8 T cell in tumor microenvironment. On the other hand, the Chicago

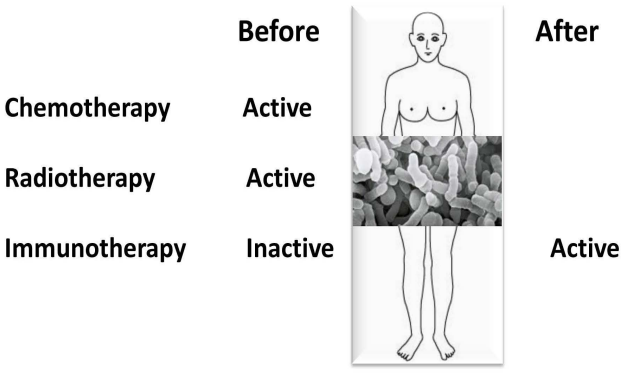

Figure 1. Differences among chemotherapy, radiotherapy, and immunotherapy

Table 1. Immunotherapy and microbiota

Immunotherapy	Disease	Microbiota	Human/Mouse	References
CTLA4	Melanoma	*B. fragilis/B. thetaiotaomicron*	Mouse → Human	Vétizou *et al*., 2015[31]
PD-L1	Melanoma	*Bifidobacterium*	Mouse → Human	Sivan *et al*., 2015[32]
PD-1	Melanoma	Diversity of microbiota/*Clostridia* → responder *Bacteroidia* → non-responder	Human	Gopalakrishnan *et al*., 2017[33]
Stem cell transplant	Allogeneic hematopoietic stem cell transplantation	Not particular, diversity of microbiota	Human	Taur *et al*., 2014[66]

group[32] clearly demonstrated that antitumor effect using anti-PD-L1 antibody is derived from commensal *Bifidobacterium*. In this publication, it was an interesting observation that the mice from one vendor showed strong antitumor effect, whereas the mice from another vendor did not show antitumor effect, even though these mice were of the same strain. When these mice were kept together in the same cage, antitumor effect transferred to the mice from the vendor that previously did not show antitumor effect. Therefore, the group hypothesized that the antitumor character might have been transferred via eaten stool, which contained various kinds of bacteria. Finally, they found *Bifidobacterium* is the key bacterium to determine the antitumor effect, using anti-PD-L1 antibody. As for the mechanism, it was also thought that *Bifidobacterium* stimulates DCs, and these DCs also stimulate antitumor immune response[65]. This evidence strongly suggested that microbiota are strongly correlated with antitumor effects. However, the limitation is that the evidence came from microbiota in mice.

Recently, human data were presented by MD Anderson Cancer Center at the ASCO-SITC 2017 Clinical Immuno Oncology Symposium[33]. Analysis was performed on melanoma patients treated with anti-PD-1 antibody. Comparison between responder group and non-responder group was made for microbiota identified using 16S rRNA. In the stool of responders, *Clostridia* was dominant, whereas in the stool of non-responders, *Bacteroidia* was dominant. On the other hand, one of the significant factors in the comparison between responders and non-responders was the diversity of microbiota. This phenomenon has also been reported in regard to the clinical efficacy of stem cell transplant[66]. It is of importance that diversity is strongly correlated to the CD8-positive cells infiltrated to tumor microenvironment. This means that microbiota are among the key factors for determining the clinical effect using immunotherapy, especially anti-PD-1 antibody.

It has been known that T cells play an important role in the showing of antitumor effect when using immunotherapy against cancer. We hypothesize that diversity of microbiota is significantly important for potent antitumor effect, because T cells are prepared for the pathogens. The number of pathogens present induce proportionate amounts of TCR, which are recognized by T cells. Gut microbiota somehow enter into human tissues from the gastrointestinal tract and travel via the blood. Innate immune responses react immediately and kill the bacteria in healthy subjects. If the immune response is weak in expelling the invading bacteria because of immunodeficient condition, live bacteria circulate in the blood: the condition of bacteremia. This is not very common in patients with normal immunity. Some of the bacteria are phagocyted by DCs or macrophages, and transfer their information as sequences of peptides restricted by MHC to adaptive immunity such as T cells and B cells. T cells are differentiated to cytotoxic T lymphocytes (CTL) against target cells, namely cellular immunity, and finally to memory T cells. B cells are differentiated to plasma cells against target molecules, namely humoral immunity, and finally to memory B cells. If a target pathogen invades the body, these memory T cells and memory B cells are immediately activated and get rid of the target. If microbiota are diverse, there are probably many pathogens in the body. This means that many kinds of TCRs are stimulated, and defend well against the invading targets. However, questions are raised because during that time, these targets are bacteria, not tumor. It has been well known that TCR is not sufficiently unique to recognize the target molecule, which is the complex binding target-derived peptides and MHC molecule. TCR often shows cross-reactivity, which is one of the major reasons autoimmune disease is induced etiologically. Type 1 DM sometimes occurs after infection with

common virus. It is thought that T cells against these viruses also may recognize the Langerhans cells, and afterwards these T cells attack and kill the pancreatic Langerhans cells. Recently, the most potent antigen against tumor is thought to be a neoantigen, which is derived from the mutations in the tumor. We hypothesize that T cells induced by microbiota might cross-recognize the tumor neoantigen. The presence of many pathogens provides a good opportunity for cross-recognition of the tumor antigen, including neoantigens *in vivo*.

Future directions

To clarify this hypothesis and the relationship between microbiota and clinical response, our group attempted to establish a microbiota database information bank system to accumulate information on microbiota. Big data might be needed for accurate evaluation and to utilize them in development of predictive markers, novel diagnoses, and modalities. There are many publications about immune competencies; however in clinical settings, no conclusive definition has yet been determined as to what the immune competencies by the host are. We believe that the tumor microenvironment, especially immune cell infiltration, is one of the significant immune competencies by the host, because it strongly correlated with the clinical effect of immunotherapy, immune checkpoint inhibitor therapy[67–71]. It is strongly expected that a predictive marker might be discovered from the big data on the relationships between immunotherapy and microbiota, and will provide us an opportunity to promote the clinical efficacy by altering the microbiota. Finally, we also expect the development of novel cancer vaccines targeting microbiota using meaningful databank information to induce potent CTLs with high diversity.

Conflict of interest

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of their article.

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REVIEW ARTICLE

Obesity and inflammatory skin diseases

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ABSTRACT

Obesity has become a significant public health problem since it may cause many chronic diseases, including type 2 diabetes, liver diseases, cardiovascular diseases, and some cancers. Recent studies show that obesity is a major risk factor for the development of inflammatory skin diseases, including eczema, atopic dermatitis, and psoriasis. Inflammatory cytokines produced from adipose tissue and activation of innate immunity are considered as important factors in obesity-induced inflammation. However, the molecular mechanisms by which obesity affects the development of inflammatory skin diseases are not well understood. In this review, we will discuss the relationship between the underlying mechanisms linking obesity and inflammatory skin diseases based on the latest researches.

***Keywords**: obesity; psoriasis; inflammation; adipokine; high fat diet; fatty acids*

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Introduction

The prevalence of obesity has increased markedly in most developed countries over the past two decades[1]. By the year 2000, around two-thirds of adults in the United States were overweight (body mass index (BMI) 26–30) or obese (BMI >30)[2], and there were 300 million of obese adults worldwide. The incidence of type 2 diabetes increased proportionally during this same time period; this trend is presumed to be a direct result of the obesity epidemic[3]. Even though obesity has long been recognized as a vital cause of diabetes and cardiovascular diseases, the impact of obesity on the skin has received minimal attention. However, recent studies have gradually revealed the close relationships between obesity and various skin diseases and skin homeostasis. For example, the epidermal barrier is reported to be impaired in obesity, so that obese individuals show increased transepidermal water loss (TEWL) and dry skin[4]. Obese individuals also have larger skin folds and sweat more profusely when overheated[5]. Obesity inhibits lymphatic flow[5] and alters collagen formation[6]. The delayed-type hypersensitivity response is increased in obese individuals and decreases with weight reduction[7], which may be related to an alteration in the production of cytokines by adipocytes.

This article aims to highlight the association between obesity and dermatologic conditions. We review the impact of obesity on inflammatory skin diseases, including eczema, atopic dermatitis and psoriasis.

Epidemiology of the association between obesity and inflammatory skin diseases

Eczema and atopic dermatitis

Eczema, the most common inflammatory skin disease, is estimated to have affected 245 million people globally in 2015[8]. In the United States, it affects about 10%–30% of people. Eczema, also known as dermatitis, is a clinical and

histopathological pattern of skin inflammation that presents as pruritus, skin dryness and erythema[9]. Eczema may result in skin lichenification, and severe eczema is the most common cause of erythroderma. Quality of life for patients and their families may be considerably reduced and occupational issues may add to the financial consequences[10].

There are some reports regarding the association between obesity and eczema[11,12]. Silverberg *et al*. reported that BMI-for-age percentiles (BMIP) of 50 to 94 and greater than or equal to 95 are each associated with higher odds of eczema compared with BMIP of 5 to 49[11]. The incidence of moderate to severe eczema is higher among individuals with BMIP of 50 to 94 and those with BMIP greater than or equal to 95 than among those with BMIP of 5 to 49. There is a significant interaction between race/ethnicity and BMIP in multivariate regression models of eczema severity, such that BMIP remains significant in Hispanics, non-Hispanic whites, Pacific Islanders/Alaskan Natives, Asians, and multiracial/other but not in non-Hispanic blacks or American Indians[11]. Thus, epidemiologically, it is suggested that the incidence of eczema correlates with BMI.

The above two reports[11,12] regarding the association between eczema and obesity, however, include many atopic dermatitis patients in the analysis. Therefore, in the next paragraph, we will go into the details about the association between atopic dermatitis and obesity.

Atopic dermatitis is a chronic, relapsing, inflammatory skin condition characterized by itch[13–16], which affects 20%–30% of schoolchildren and 5%–10% of adults in the UK[10]. Topical therapy with emollients, corticosteroids, and calcineurin inhibitors remains the mainstay of treatment. For more severe cases, current therapeutic options remain limited. As a result, atopic dermatitis still places a significant quality-of-life and financial burden on society and health care systems worldwide[10,16].

There are more than 30 studies about the relationships between obesity and atopic dermatitis[17]. In general, it is reported that patients who are overweight or obese have higher odds of atopic dermatitis than normal weight patients. In sensitivity analyses, children who are overweight or obese and adults who are obese have higher odds of atopic dermatitis. The association is also significant in North America and Asia but not Europe. As the phenotype of atopic dermatitis is different between the regions[18], the contribution of obesity to the development of atopic dermatitis may be different between the regions. Although the underlying mechanisms by which obesity causes eczema and atopic dermatitis remain largely unknown, various mechanisms have been proposed, including obesity-induced exacerbation of skin inflammation, which will be discussed later.

Psoriasis

Psoriasis encompasses a group of related inflammatory skin diseases that affect up to 3% of the population[19]. These diseases are heritable and over 40 genetic susceptibility loci have been identified, many of which are involved in antigen presentation, cytokine signaling, or innate antimicrobial responses. The most common presentation of psoriasis is plaque psoriasis but the disease is clinically heterogeneous in its manifestations and natural history depending on the age of the patient, environmental triggers, and the sites affected[10]. Treatments include topical agents for disease of limited extent, phototherapy (UVB and PUVA), and systemic therapy (methotrexate, cyclosporine, acitretin and fumarates) for more extensive disease, and biological treatment (inhibitors for tumor necrosis factor α, IL-23 and IL-17) for severe resistant psoriasis[10].

Many reports cite an association between obesity and psoriasis. From 1980 to 2012, sixteen observational studies were conducted with a total of 2.1 million study participants[20]. The frequency of psoriasis varied significantly in relation to BMI (OR = 1.6 and 1.9 for overweight and obese patients, respectively)[21]. Prospective data on 78,626 women in a nurses' health study found that weight gain placed individuals at an increased risk for the subsequent development of psoriasis. Moreover, the incidence of psoriasis was linearly associated with the BMI, with the greatest relative risk of 2.69 for patients with a BMI of 35 or more compared to patients with a BMI of 21 to 23. The risk of psoriasis at 18 years of age was also linearly associated with BMI[22]. It is likely that obesity predisposes individuals to the development of psoriasis. Obesity is also associated with the severity of psoriasis. It has been reported that BMI and psoriasis area and severity index (PASI) are correlated[23]. Unlike other inflammatory diseases, psoriasis has been reported to be associated with obesity in all regions of the world.

Mechanistic links between obesity and skin inflammation

Although there are considerable epidemiological data, as described above, the mechanisms linking obesity with skin inflammation are diverse, and there are many unclear points. In the next section, we will

introduce these mechanisms that are currently under consideration.

Obesity changes cell composition in adipose tissues

As discussed above, inflammatory skin diseases are generally exacerbated by obesity, but the mechanism of this exacerbation has not been fully elucidated. It has been reported, however, that changes in cell composition in adipose tissues are important for the development of systemic diseases such as diabetes in obesity[24]. Adipose tissues are composed not of adipocytes alone but rather of a variety of other cell types, collectively termed the stromal vascular fraction (SVF). This fraction includes mesenchymal stem cells, vascular endothelial cells, nerve cells, macrophages, T cells and B cells. In 2003, pioneering studies by Xu *et al*. and Weisberg *et al*. reported that obesity is associated with significant increases in the proportion of macrophages in the SVF in both visceral and subcutaneous adipose tissues[25,26]. Flow cytometric analysis has shown that macrophages account for approximately 40% of the SVF in obese rodents, whereas it accounts for only 10% in lean littermates. The gene expression profile of adipose tissues from multiple obese mouse models demonstrates that macrophage-related genes are upregulated in obese animals[26]. Recruitment of macrophages into adipose tissues is an early event in obesity-induced adipose inflammation. The monocyte chemoattractant protein-1 (MCP-1)/CCL2, one of the major chemoattractants for macrophages via CCR2, is secreted primarily by macrophages and vascular endothelial cells, and also by adipocytes[27]. Adipose tissue macrophages (ATMs) express CCR2 and recruit additional monocytes/macrophages, promoting a feed-forward process[26,27]. As it is reported that macrophages play an important role in inflammatory skin diseases such as contact dermatitis and psoriasis[28,29], an increase in ATM in adipose tissues in the skin (subcutaneous and intradermal adipose tissues)[30] may contribute to the deterioration of inflammatory skin diseases.

Adipokines and inflammation

Presently, the hypothesis that adipose tissues have an endocrine function[31] is supported by the findings that adipocytes secrete a variety of mediators, namely adipokines, which are involved in the inflammatory network. These mediators include leptin, adiponectin, plasminogen activator inhibitor (PAI)-1, interleukin (IL)-6 and tissue necrosis factor (TNF)-α[32] (**Figure 1**). Particularly, not only increased cytokine production but also impaired cytokine catabolism seem to be determinants of obesity-related inflammatory status[33].

Adipokines and psoriasis

As mentioned above, adipokines have the function of regulating inflammation. Several basic and clinical studies have been published on adipokines' role in the pathology of inflammatory skin diseases (especially psoriasis).

Leptin is one of the main adipose-derived cytokines. Leptin has been investigated primarily for its

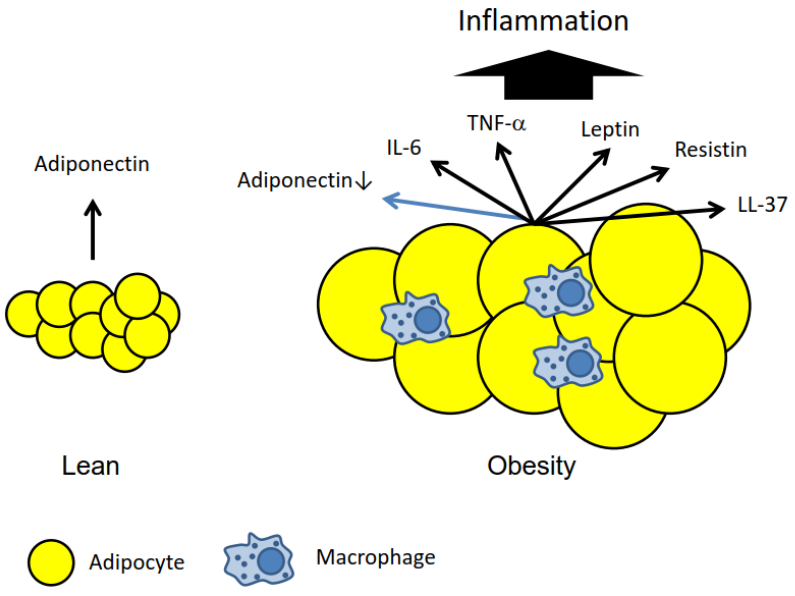

Figure 1. Hypertrophic adipose tissue produces inflammatory cytokines. Obesity increases the proportion macrophages in adipose tissue. In obesity, production of inflammatory cytokines from adipose tissue increases, causing inflammation.

role in controlling energy homeostasis by regulating appetite[34]. Leptin is also essential for cell-mediated immunity. Previous studies reported that $CD4^+$ T cells are hyporeactive in leptin-deficient mice[35]. In psoriasis patients, leptin levels showed correlation with psoriasis severity[36]. Furthermore, leptin-deficient (ob/ob) mice exhibit impaired IL-17A and IL-22 mRNA expression as well as reduced epidermal hyperplasia in an imiquimod (IMQ)-induced psoriasis model[37]. *In vitro*, leptin induces proliferation and production of several pro-inflammatory proteins on human keratinocytes[37]. These results suggest that leptin may exacerbate the condition in psoriasis.

Adiponectin is an anti-inflammatory cytokine mainly produced by adipocytes. Low serum levels of adiponectin have been reported in several chronic diseases, for example obesity and psoriasis[38]. High levels of cytokines (eg. TNF-α and IL-6) may decrease adiponectin production in patients affected by inflammatory diseases[38]. Low serum levels of adiponectin have been observed in obese psoriatic patients as compared with non-obese psoriatic patients[39,40]. Shibata *et al*. reported that mice with adiponectin deficiency are present with severe psoriasiform skin inflammation with increased infiltration of IL-17-producing T cells[41]. Adiponectin suppresses IL-17 synthesis via AdipoR1 on murine T cells. Adiponectin levels in skin tissue as well as in subcutaneous fat are decreased in psoriasis patients[41]. IL-17 production from human CD4- or CD8-positive T cells is also suppressed by adiponectin[41].

Zhang *et al*. reported that LL-37 (cathelicidin), one of the inducers of psoriasis[42], is produced from the intradermal adipocytes under *Staphylococcus aureus* infection[42]. This LL-37 production is inhibited by an adipocyte-differentiation inhibitor (peroxisome proliferator-activated receptor γ inhibitor). Interestingly, in the mice with high fat diet (HFD)-induced obesity, the numbers of intradermal adipocytes and their production of LL-37 are increased[43]. Therefore, the immunological and metabolic alterations associated with obesity may be associated with the pathophysiology of psoriasis.

Obesity induces dysfunction of skin barrier

Obesity is associated with a number of significant changes in skin barrier functions. Obese individuals showed significantly increased TEWL, skin blood flow and skin color (*i.e.* redness) as compared to a control group in a study examining clinically unaffected skin in the middle of the flexor side of one forearm[44], suggesting a fundamentally altered epidermal barrier. Consistently, in obese mice, TEWL is also increased[45]. Their numbers of keratinocytes are reduced, and their epidermis has a flattened and thinner appearance in obese mice[45]. The epidermal structure is exacerbated by factors in the obese environment as demonstrated by altered E-cadherin localization and impaired cell–cell adhesion[45]. A dysfunction of the skin barrier allows for more opportunities for antigen sensitization, which can cause various allergic diseases. In fact, obesity is a risk factor not only for atopic dermatitis, but also for asthma[46].

Obesity and the inflammasome

The emergence of chronic inflammation during obesity in the absence of overt infection or well-defined autoimmune processes is a confusing phenomenon. The NOD-like receptor (NLR) family of innate immune cell sensors, for example, the nucleotide-binding domain, the leucine-rich-containing family, and the pyrin domain-containing-3 (Nlrp3) inflammasome, are implicated in recognizing certain nonmicrobially-originated "danger signals" leading to caspase-1 activation and subsequent IL-1β and IL-18 secretion. The expression levels of IL-1β and Nlrp3 in the adipose tissue correlate with body weight and insulin resistance (**Figure 2**). Ablation of Nlrp3 in mice avoids obesity-induced inflammasome activation in fat depots and the liver and enhances insulin signaling[47,48].

The inflammasome may be also important in obesity-induced exacerbation of psoriasis-like skin inflammation. Vasseur *et al*. reported that imiquimod-induced psoriasiform dermatitis is exacerbated in obese mice as compared to lean mice[49]. Scale formation and acanthosis are aggravated in correlation with increased IL-17A and IL-22 expressions in inflamed skins. Moreover, obesity is associated with the epidermal activation of caspase-1 and the cutaneous overexpression of IL-1β[49] (**Figure 2**), suggesting that obesity induces inflammasome activation in the skin.

Obesity and lymphatic dysfunction

Recent studies reported that obesity impairs lymphatic functions in both mice and humans[50,51]. For instance, obese mice have reduced ability to transport interstitial fluid via cutaneous lymphatics and have remarkably reduced trafficking of antigen-presenting cells to regional lymph nodes[52]. Obese mice have impaired lymphatic collecting vessel pumping functions[53]. These findings are supported by clinical studies reporting that obese patients have decreased clearance of macromolecules from

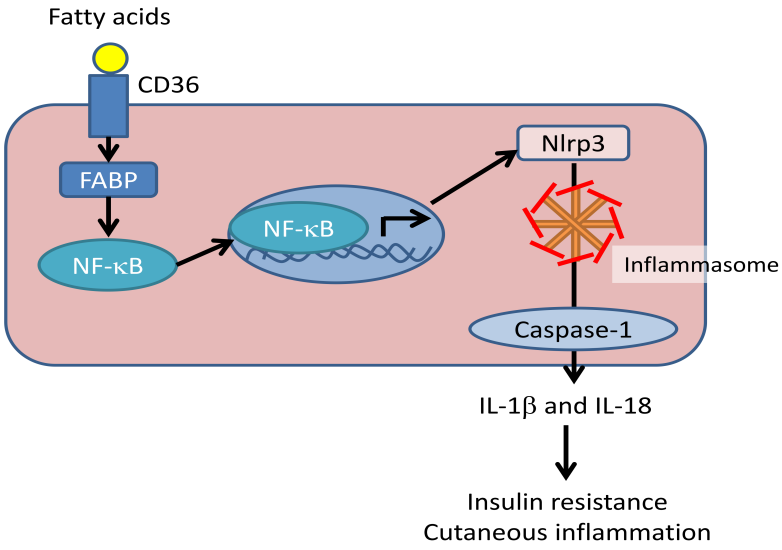

Figure 2. Fatty acids induce inflammasome activation. Fatty acids bind to CD36 and activate NF-κB. NF-κB activates Nlrp3 and the inflammasome to produce IL-1β and IL-18.

fat depots compared with normal controls, and that massively obese patients spontaneously develop lymphedema[51]. Savetsky *et al.* reported that obesity-induced lymphatic dysfunction regulates contact hypersensitivity[54]. Obese mice have impaired lymphatic functions and intensified dermatitis responses to inflammatory skin stimuli. Injection of recombinant vascular endothelial growth factor-C remarkably increased lymphangiogenesis, and lymphatic functions, while decreasing inflammation. These results propose that obesity-induced changes in the lymphatic system cause an amplified and prolonged inflammatory response in the skin.

Fatty acids and inflammation

Fatty acids, such as saturated fatty acids and trans fatty acids, are abundantly contained in HFD, which is considered a cause of obesity[55]. There are several papers that show the direct effects of fatty acids on immune functions. For example, GPR120, which is a receptor for ω3 fatty acid, is expressed in macrophages and adipocytes, and inhibits inflammation by suppressing Toll-like receptor signals and TNF signals[56]. On the other hand, CD36, which is a receptor of unsaturated fatty acid, is expressed in vascular endothelial cells and macrophages and is known to cause the production of TNF and IL-1 via NF-kB[57].

Stelzner *et al*. reported that the concentration of fatty acid is increased in the HFD-fed mice, and that the concentration of fatty acid is correlated with the severity of the imiquimod-induced psoriasis model[58]. Kanemaru *et al*. reported that saturated fatty acids activate keratinocyte to up-regulate the IL-17A downstream molecule, regenerating islet-derived 3γ, which has been suggested as a critical molecule for epidermal hyperplasia in psoriasis[59]. It is also reported that dietary fatty acids induce obesity-associated inflammasome activation in the skin and cause skin inflammation (**Figure 2**). Zhang *et al.* reported that mice fed with HFD for six months spontaneously develop detectable skin lesions[60]. In mice, langerin-positive dendritic cells are increased in both the epidermis and the dermis where they produce IL-1β and IL-18. The activation of the inflammasome depends on the concentration of fatty acids. Fatty acid binding protein 5, the transporter of fatty acids, is reported to mediate such fatty acids-induced skin inflammation, because mice without fatty acid binding protein 5 did not exhibit any of the above symptoms while receiving HFD[60] (**Figure 2**). As the fatty acid binding protein 5 was first identified as being upregulated in the psoriasis tissue, a similar mechanism may be involved in the deterioration of psoriasis caused by obesity.

There is a report that fatty acids are involved in the differentiation of Th17 cells, a critical cell subset in the pathogenesis of psoriasis[61]. Endo *et al.* reported that Th17 is increased in the spleens of obese mice. Increased fatty acid synthesis in Th17 cells was suggested as the mechanism of increased Th17 differentiation in obese mice, since inhibition of fatty acid synthesis reversed the obesity-induced increase of Th17 differentiation while the addition of fatty acids facilitated Th17 cell differentiation *in vitro*[61]. These results suggest that high intake of fatty acids can increase Th17 cell differentiation in humans,

which may explain one mechanism linking obesity and psoriasis.

Fatty acids may also be important for the survival of the cutaneous resident memory T cells (T_{RM})[62]. T_{RM} are a recently described subset of memory T cells that persist long-term in peripheral tissues[63]. T_{RM} undergo a distinct differentiation program that discriminates them from circulating T cells; this program likely evolved to populate epithelial barrier tissues—the skin, gut, lung, and reproductive tracts—with highly protective T cells specific for the pathogens most commonly encountered through each tissue[63]. T_{RM} have been directly demonstrated in psoriasis, mycosis fungoides, and fixed drug reactions[63]. Cutaneous T_{RM} express fatty acid binding proteins 4 and 5, and the number of T_{RM} are decreased in the fatty acid binding protein 4- and 5-deficient mice[63] . Unlike other memory T cells, T_{RM} actually produce ATP by β-oxidation of fatty acid. The protection against virus infection is reduced in through the fatty acid 4- and 5-deficient mice as well as β-oxidation inhibitor-treated mice, suggesting that fatty acids are essential for the protection of the host from virus infection via maintenance of T_{RM}. Fatty acid binding proteins 4 and 5 are also expressed in the human T_{RM} in psoriasis patients[62]. However, the relationships between obesity and T_{RM} are unknown; increased quantities of fatty acid due to obesity may have some effects on the T_{RM}.

Conclusion

Obesity is one of the important causal factors of many inflammatory diseases. In the skin, functional changes in both adipocytes and lymphatic vessels and epidermal keratinocytes are suspected to be involved in the obesity-induced exacerbation of skin inflammation. How obesity causes inflammation is still not well understood, but future research will reveal these mechanisms, enabling the development of new treatments for inflammatory skin diseases is expected.

Conflict of interest

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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SHORT REPORT

Therapeutic effects of cyclosporine on Hailey-Hailey disease

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Running title: Effects of cyclosporine on Hailey-Hailey disease

ABSTRACT

Hailey-Hailey disease is an autosomal dominant hereditary skin disease. Severe cases are often difficult to treat. We report a recent case that was successfully treated using cyclosporine. The case is described from the aspects of treatment and gene mutation.

Keywords: *Hailey-Hailey disease; cyclosporine; ATP2C1; treatment*

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Introduction

Hailey-Hailey disease (HHD) is an autosomal dominant hereditary skin disease associated with vesicles, erosions and crusts on the intertriginous after middle-age. The lesions usually heal without scarring. Sunlight, heat, sweating and friction often aggravate the disorder. Recent studies revealed many types of gene mutations. The responsible gene for HHD is *ATP2C1*, which encodes human secretory pathway Ca^{2+}/Mn^{2+}-ATPase protein 1 (SPCA1), a Ca^{2+} pump expressed in the Golgi apparatus[1]. We also reported the case of 50-year-old Japanese male who had a novel heterozygous c.1627G>T transition on exon 18 of *ATP2C1*, causing premature termination (PT) at amino acid 543 (p.Gly543X)[2]. Over 150 pathological mutations have been identified throughout *ATP2C1*[3].

Regarding treatment, severe cases are often difficult to treat. We recently successfully treated a case using cyclosporine. The case is described from the aspects of treatment and gene mutation.

Case Presentation

A 70s-year-old Japanese female had a history of recurrent erythematous lesions with erosions on the axillae and genital region, especially in summer. Approximately 30 years ago, she had been diagnosed with HHD by a dermatologist. Her mother had similar skin lesions, but the details were unclear. Her two daughters have no such skin lesions. Although topical steroids, etretinate (40 mg/d) and/or prednisolone (10 mg/d) were prescribed, she developed difficulty in walking due to painful inguinal erosions and was admitted to our department. Her skin lesions included erosions with pustular discharge and erythematous lesions with vesicles on the inguinal area (**Figure 1**).

Her blood examination results were almost within normal limits. Histopathological examination of vesicular erythema on the back revealed the separation of keratinocytes (acantholysis) at the suprabasal layers of the epidermis, which resembled a “dilapidated brick wall” (**Figure 2**).

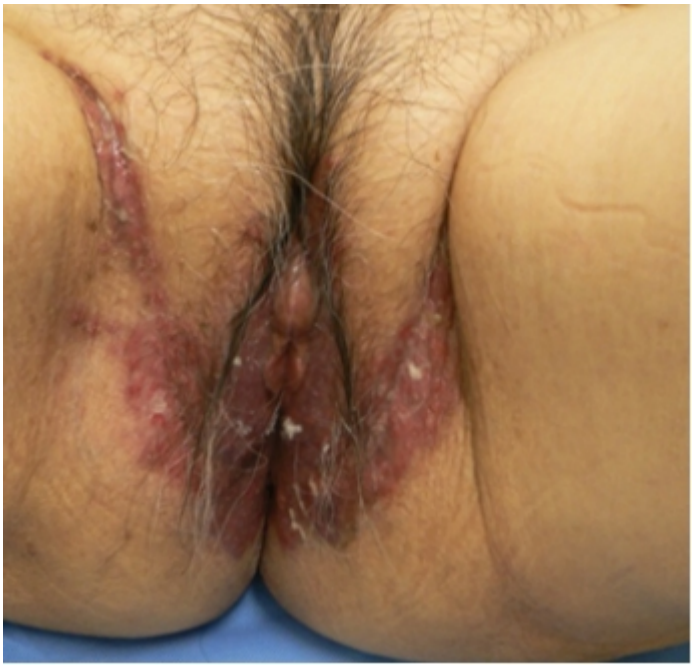

Figure 1. Pustular discharge and erythematous lesions**.**

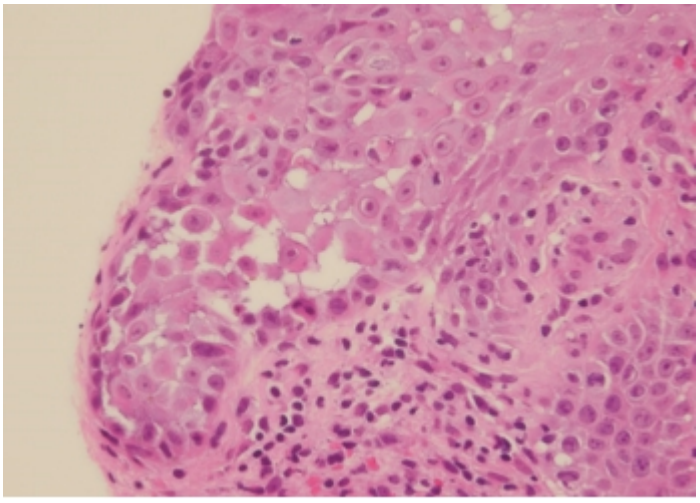

Figure 2. Acantholysis at the suprabasal layers of the epidermis**.**

Direct immunofluorescence staining was negative. By genetic analysis of the peripheral blood, a novel heterozygous mutation c.2305delG (p.S769Afs*3) on exon 24 of *ATP2C1* was identified and the diagnosis of HHD was confirmed.

After hospitalization, she was administered cyclosporine (3 mg/kg/d) and minocycline (100 mg/d) for 8 days. The lesions gradually improved and fully epithelialized 15 days after the start of treatment (**Figure 3**).

Figure 3. Skin lesion 15 days after the start of treatment**.**

At the outpatient clinic, a topical steroid was effective for a small erosion on the genital area, and a small amount of cyclosporine was able to control the skin lesions when symptoms worsened.

Discussion

The standard treatments for HHD are directed toward the specific symptoms of each patient. Specific therapies depend upon several factors, including the extent and severity of the disease, and patients' age. It is essential to avoid triggers such as sunburn, sweating and friction. Topical corticosteroid and topical antibiotics may be effective for mild cases, whereas more serious cases may require systemic antibiotics or stronger topical corticosteroid, although some of them may cause severe side effects. The treatments for 43 cases reported in Japan between 2002 and 2019 are summarized in **Table 1**.

Treatment	**Cases**
Topical corticosteroid	31
Topical Vitamin D3	9
Systemic corticosteroid	8
Etretinate	5
Topical tacrolimus	3
CO_2 laser	2
Split skin grafting	1
Surface skin grafting	1
Cyclosporine	1

Table 1. Summary of main treatments for 43 cases reported in Japan between 2002 and 2019. Secondary or more treatments for infection were excluded from this table

As investigational therapies, botulinum toxin[4], oral glycopyrrolate and several others have been reported to reduce the effects of sweat glands. For refractory HHD, additional medications have been tried, including vitamin A derivatives (retinoids), such as acitretin and etretinate, and drugs that suppress the immune system such as alefacept[5] or tacrolimus. Further studies are necessary to clarify the long-term safety and effectiveness of such drugs.

Very recently, Ichikawa reported a case of HHD with a novel missense/in-frame deletion mutation in *ATP2C1* successfully treated using cyclosporine[6]. In the practical treatment for intractable inflammatory diseases as well as autoimmune diseases, cyclosporine is often used for initial therapy and/or

maintenance therapy[7-9]. As cyclosporine may block the calcineurin/NFAT/interleukin-6-mediated suppression of *ATP2C1* transcription[10,11], it effective for refractory conditions

Conflict of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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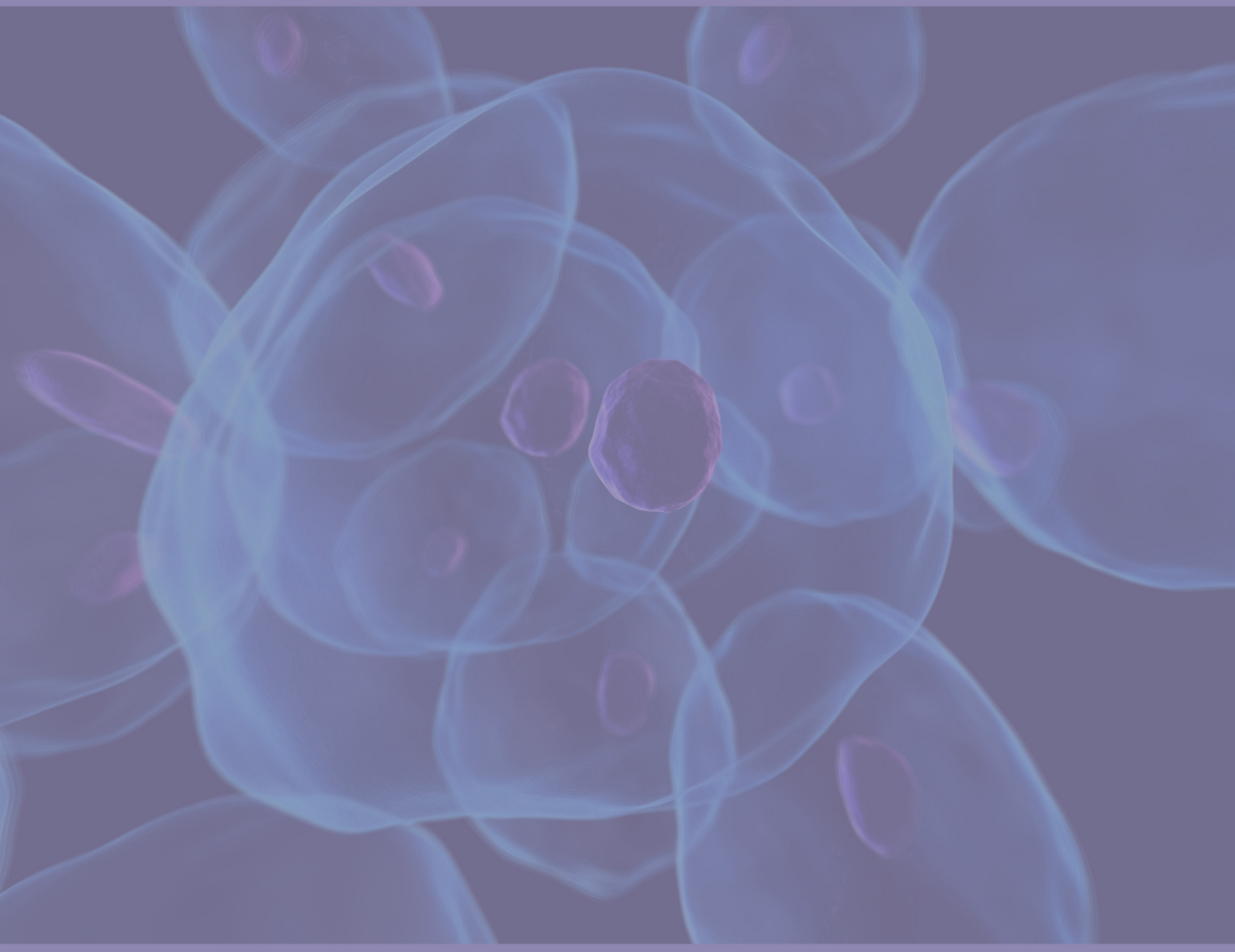

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