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Original Article

Staphylococcus aureus from atopic dermatitis skin alters cytokine production triggered by monocyte-derived Langerhans cell

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ABSTRACT

Background: Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases. The skin of patients with AD presents as a disbalance of the microbiome with a strong colonization by *Staphylococcus aureus*, which positively correlates with the severity of the disease. However, the effect of colonized *S. aureus* on the skin immune system has not been fully elucidated.
Objective: The aim of this study is to explore whether *S. aureus* isolated from AD skin is able to skew T cell responses via Langerhans cells (LC) as compared to a standard strain of *S. aureus* and *S. epidermidis*.
Methods: We prepared monocyte-derived LC (MoLC) from healthy controls and patients with AD, and stimulated MoLC with a standard strain of *S. aureus* NCTC8325, *S. aureus* TF3378 isolated from AD skin, or *S. epidermidis*. Stimulated MoLC were co-cultured with autologous $CD4^{pos}$ T cells and then T cell responses were analyzed by T cell polarization assays, cytokine analysis and real-time PCR.
Results: MoLC stimulated by *S. aureus* TF3378 induced significantly high and rapid proliferation of T cells as compared to those by *S. aureus* NCTC8325 and *S. epidermidis*. Cytokine productions from T cells cultured with *S. aureus* TF3378-stimulated MoLC showed significantly high amounts of IL-2 and less IFN-γ production with imbalanced Th1/Th2 (decreased *TBX21*/*GATA3* ratio) mRNA expression. The T cell proliferation with increased IL-2 production via *S. aureus* TF3378-stimulated MoLC was diminished by treatment of proteinase K.
Conclusion: S. aureus TF3378 on AD skin can skew T cell responses via LC toward imbalanced Th1/Th2 skin immunity.

1. Introduction

The skin is consistently exposed to environmental factors including the skin microbiome. Healthy skin maintains its homeostasis by recognizing commensals and pathogens via receptors of the innate immune system, such as Toll-like receptors (TLRs) [1]. In patients with atopic dermatitis (AD), a chronic inflammatory skin disorder affecting 2 to 10% of adults in developed countries [2,3], persistent skin colonization with *Staphylococcus aureus* [4] was detected. The increase of *S. aureus* during eczema flare-up was demonstrated by Staphylococcus genomic analysis [5]. Moreover, biologically active TLR2 ligand, lipoteichoic acid derived from *S. aureus* was detected in impetiginized skin from patients with AD [6]. It is also reported that patients with AD have significantly more frequent bacterial infection by *S. aureus* than individuals without AD [7]. These findings suggest that an imbalance of the skin microbiome is strongly associated with AD pathogenesis.

Langerhans cells (LC) represent the principal professional antigen presenting cells and express several surface TLRs to sense danger signals from the external environment [8]. Mature LC can activate and polarize T cells to Th1, Th2, Th17 or regulatory T cells

Abbreviations: ADA, topic dermatitis; *ACTB*, beta actin; *FOXP3*, forkhead box P3; *GATA3*, GATA binding protein3; HC, healthy controls; LC, Langerhans cells; MLR, mixed lymphocyte reaction; MoLC, monocyte derived Langerhans cells; rFI, relative fluorescence index; *RORC*, RAR-related orphan receptor C; *TBX21*, T-box21; Treg, regulatory T cell; TLR, toll-like receptor; 7-AAD, 7-amino-actinomycin D.

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(Treg) depending on microenvironmental factors to which the LC might contribute [9]. Therefore, LC have been recognized to have a pivotal role in bridging innate and adaptive immunity [10]. The skin microbiome does not just represent static bacterial presence on the skin surface, but is able to modify the local inflammatory cytokine milieu and skin immunity via dendritic cells [11,12]. Indeed, *S. epidermidis*, a skin commensal mostly detected from healthy individuals, was able to restore IL-17 production in germ-free mice with low IL-17 to maintain cutaneous immune homeostasis [12].

However, skin immune responses via activated LC by different strains of *S. aureus*, especially clinically isolated strains from AD skin, have not been studied. We hypothesized that colonized *S. aureus* on AD skin may induce distinct T cell responses toward the Th2 prone AD cytokine milieu as compared to standard *S. aureus* strains.

2. Materials & methods

This study was approved by the local ethics committee of Hiroshima University (No. E-57) and performed in accordance with the Declaration of the Helsinki principle. Informed consent was obtained from all participants in this study.

2.1. Patients

This study was conducted with 24 healthy controls (HC) and 13 patients with AD, who were diagnosed according to the criteria by Japanese Dermatological Association [13]. The detailed backgrounds of participants are follows; HC vs AD, indicated as mean ± SEM; age, 29 ± 2 vs 30 ± 2 years; serum IgE, 133 ± 59 vs 13667 ± 5105 IU/ml; serum thymus and activation-regulated chemokine (TARC), 265 ± 24 vs 3744 ± 1240 pg/ml.

2.2. Reagents

The medium used for the culture was RPMI1640 supplemented with 10 % heat-inactivated FBS, 1 % penicillin G and streptomycin obtained from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant human GM-CFS, recombinant human IL-4 and human TGF-β1 were from R&D systems (Minneapolis, MN, USA). TLR1/2 ligand Pam3CSK4 was from InvivoGen (San Diego, CA, USA). Details of antibodies for flow cytometry used are given in Table S1. 7-amino-actinomycin D (7-AAD) was from Becton Dickinson (San Jose, CA, USA). SYBR Green was obtained from TaKaRa Bio (Shiga, Japan).

2.3. Preparation of S. aureus and S. epidermidis

Five different strains of *S. aureus* TF3378, TF2962, TF2868, TF2820, NCTC8325 and *S. epidermidis* ACTT12228 were used in this study. *S. aureus* TF3378, TF2962, TF2868 and TF2820 were clinically isolated from the skin surface of different patients with AD. *S. aureus* NCTC8325 was a laboratory control strain [14] and used as control for clinical isolates [15]. The sequence types (ST) of *S. aureus* strains are follows: TF3378 (ST59), TF2962 (ST59), TF2868 (ST121), TF2870 (ST20), and NCTC8325 (ST8). They were grown in Tryptic soy broth (TSB) (Becton Dickinson) for 24 h at 37 °C with shaking, and washed by PBS twice. They were adjusted by Optical Density (OD) at 660 nm to 1.0 followed by calculations of their colony-forming units (CFU) (Figure S1). Both *S. aureus* strains and *S. epidermidis* were heat-inactivated for 1 h at 57 °C. Proteinase K treatment and extraction of cell wall-associated proteins by lysostaphin were performed as described respectively [16,17]. Cell wall-associated proteins were loaded onto 7.5% SDS-PAGE, then silver stained by using 2D-silver stain II (Cosmo Bio, Tokyo, Japan).

2.4. Generation and stimulation of monocyte-derived Langerhans cells

$CD14^{pos}$ monocytes were sorted using the EasySep™ Human monocyte enrichment kit (StemCell Technologies, Vancouver, Canada) from peripheral blood mononuclear cells. The purity of $CD14^{pos}$ monocytes was >95 % as determined by flow cytometry (data not shown). Monocyte-derived Langerhans cells (MoLC) were generated by culturing monocytes with GM-CFS (500 IU/ml), IL-4 (500 IU/ml) and TGF-β (25 U/ml) for 7 days, as described previously [18]. For stimulation of MoLC, 10^6 cells/ml were cultured in 24 well plates for 24 h at 37 °C with or without *S. aureus, S. epidermidis* (OD660 = 0.1) and Pam3CSK4 (1 μg/ml). The stimulated MoLC were subjected to flow cytometric analysis and were then used for the following experiments.

2.5. Mixed lymphocyte reaction (MLR)

$CD4^{pos}$ T cells were isolated from peripheral blood mononuclear cells of each HC and patients with AD using an EasySep™ Human $CD4^{pos}$ T cell isolation kit (StemCell Technologies). The purity of the $CD4^{pos}$ T cells was > 98 % as determined by flow cytometry (data not shown). The purified $CD4^{pos}$ T cells were kept frozen in the Cellbanker®1 medium (TaKaRa Bio) at −80 °C until the MLR experiments. MoLC were cultured with or without *S. aureus, S. epidermidis* and Pam3CSK4 for 24 h, then washed and subsequently sorted by $CD1a^{pos}$ by FACSAria™ (Becton Dickinson). Sorted $CD1a^{pos}$ MoLC (10^5 cells/well) were co-cultured with autologous $CD4^{pos}$ T cells (10^6 cells/well) from the same individuals in 200 μl/well on 96 well plate for 7 days. Supernatants were collected and analyzed for cytokine production. For proliferation assays, $CD4^{pos}$ T cells after co-culture were sorted by FACSAria™ to determine the number of accurate proliferated T cells. In some experiments, $CD4^{pos}$ T cells were stained by the CellTrace™ CFSE cell proliferation kit (Thermo Fisher Scientific) before co-culture, then divided T cells were analyzed by flow cytometry after 4 and 7 days of the co-culture.

2.6. cDNA preparation and quantitative real-time PCR

For gene expression analysis, $CD4^{pos}$ sorted T cells after co-culture were collected by FACSAria™ and RNA was extracted by the RNeasy Mini Kit (Qiagen, Tokyo, Japan). cDNA was prepared using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Gene expression was determined by SYBR Green-based quantitative real-time PCR (qRT-PCR) using the Applied Biosystems 7900HT Fast Real-Time PCR System (ABI, Foster City, CA, USA). Cycling conditions were 95 °C for 15 s and 62 °C for 1 min for 40 cycles. The following primers were purchased from TaKaRa Bio; T-box21 (*TBX21*; Primer ID; HA163073), GATA binding protein3 (*GATA3*; HA190606), RAR-related orphan receptor C (*RORC*; HA230921), forkhead box P3 (*FOXP3*; HA173697), beta actin (*ACTB*; HA067803). Results were normalized to *ACTB* and relative mRNA levels were calculated using ExpressionSuite Software (ABI).

2.7. Flow cytometry

Expression of surface markers on MoLC was evaluated by staining with fluorescent-conjugated mAb described in Table S1. MoLC were gated by the expression of $CD1a^{pos}$ after dead cell exclusion by 7-AAD. Surface markers were measure by the Attune® Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA, USA) or FACSAria™ (Becton Dickinson) and analyzed with FlowJo V7.6.5 (TreeStar, Ashland, OR, USA). The relative fluorescence index (rFI) was calculated as follows: (Mean fluorescence intensity (MFI) $_{\text{surface marker}}$ − MFI $_{\text{isotype}}$) / MFI $_{\text{isotype}}$.

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