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Dendritic cells produce inflammatory cytokines in response to bacterial products from Staphylococcus aureus-infected atopic dermatitis lesions

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Abstract

Atopic Dermatitis (AD) patients often acquire secondary skin infections resulting in increased inflammation. The increased inflammation occurs through the activation of multiple cell types including dendritic cells (DC). In this study, we investigated the activity of soluble products present in infected AD lesions by measuring the ability of patients' wash fluids from a quantitative culture of lesions to activate DC. We found that wash fluid derived from AD lesions induced cytokine production by murine bone marrow-derived DC, including IL-1β, IL-6, IL-10, and tumor necrosis factor-α. The lipoprotein lipoteichoic acid (LTA) from *Staphylococcus aureus* was implicated as a potent stimulus in the wash fluids as only wash fluid samples that contained LTA exerted this activity, and exogenous LTA triggered similar DC cytokine activation. Wash fluid- and LTA-stimulated DC cytokine production required MyD88, but not the platelet-activating factor receptor (PAF-R), despite the ability of LTA to function through this receptor in keratinocytes. Thus, our results support a role for DC in the worsening of AD inflammation due to secondary bacteria infections.

Keywords

Dendritic cells; *Staphylococcus aureus*; atopic dermatitis; lipoteichoic acid; Interleukin-6; Tumor necrosis factor-alpha; platelet-activating factor

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Introduction

Staphylococcus aureus infections are known triggers for skin inflammation and can modulate immune responses [1,2]. Patients with AD are particularly susceptible to staphylococcal skin infections, which associate with the worsening of their skin disease [3,4]. Although the mechanisms by which staphylococcal bacteria can worsen AD are not yet clear cytokine production following direct infection or interaction with bacterial products by keratinocytes or immune cells appears to play an important role [5,6]. Potential staphylococcal mediators that could worsen AD include the cell wall lipoprotein lipoteichoic acid (LTA) that can act as an agonist for the Toll-like receptor 2 (TLR2) [7] as well as the Platelet-activating factor receptor (PAF-R) [8,9].

Dendritic cells are a morphologically and functionally defined family of leukocytes that serve as highly specialized antigen-presenting cells in the skin [10,11]. In keeping with their role in immune surveillance, DC express various pattern-recognition receptors including TLRs that allow them to interact with microbial products in their environment [7]. In response to microbial products, DC produce an array of pro-inflammatory cytokines including IL-1β, IL-6, and TNFα to activate an immune response. However, they also limit the immune response through production of anti-inflammatory cytokines such as IL-10. From their ability to regulate immune processes, DC have been implicated in the pathogenesis of AD [12].

Recently, it has been demonstrated that high levels of LTA can be found in secondarily impetiginized AD lesions [13]. Quantitative bacterial culture techniques revealed that almost one third of *S. aureus*-infected AD lesions expressed microgram/cm^3 levels of LTA. High levels of inflammatory cytokines such as IL-6 and TNF-α were also found in these skin lesions. The amounts of LTA and pro-inflammatory cytokines correlated with both amounts of *S. aureus* bacteria found in AD lesions and levels of clinical inflammation [13].

In the current study, we investigated whether secondary bacteria infection produces biologically-active products in infected AD lesions which can modulate host immune responses by activating DC. We were able to demonstrate that small amounts of wash fluid derived from infected AD lesions can trigger cytokine production in murine bone marrow-derived DC (BMDC). The activation of BMDC was MyD88 dependent. The data suggest that lipoprotein LTA is an important component in AD lesion wash fluids inducing DC activation. Furthermore, MyD88 not the PAF-R plays an important role mediating signaling by soluble products in the wash fluids. These findings implicate the host immune response induced by DC in exacerbating AD during staphylococcal colonization and infection.

Methods

Mice and Cells

C57BL/6 mice (age 6–8 wk) were purchased from The Jackson Laboratory. *Paf-r*$^{-/-}$ mice were a kind gift from Professor Isshi (University of Tokyo). *Myd88*$^{-/-}$ mice were a kind gift of Dr. Kunkel (University of Michigan). Mice were housed under specific pathogen-free conditions at the Indiana University or University of Michigan's School of Medicine. All procedures were approved by the Animal Care and Use Committees of Indiana University and the University of Michigan Schools of Medicine. Murine BMDC were prepared as previously described [14].

Reagents

DC were treated with the following reagents: 100 ng/mL LPS (Sigma-Aldrich), 10 μg/mL LTA (or as described in figure legends; Invivogen), and 1-hexadecyl-2-N-methylcarbamoyl glycerophosphocholine (CPAF; Sigma-Aldrich). Polymyxin B was a kind gift of Dr. O'Roirdan (University of Michigan) and was originally obtained from Calbiochem. For DC maturation, 10 ng/mL recombinant GMCSF (Peprotech) was added as previously described [14].

Atopic Dermatitis Subjects

Subjects with AD diagnosed using criteria of Hanifen and Rajka [15] were enrolled in these studies approved by the Indiana University Institutional Review Committee. The experimental procedures were previously described [13]. Subjects enrolled into the study underwent a clinical assessment of a clinically-infected lesion of dermatitis using the Eczema Area and Severity Index (EASI) [16]. Wash fluid was removed from the lesion and the subject treated with appropriate therapy, to include oral cephalexin antibiotic. Following two weeks, the subjects returned and the procedures were repeated.

Quantitative Analysis of Dermatitis Lesions

Wash fluid derived from lesions was removed from a 2.5 cm diameter polypropylene chamber as previously described [9,13] using the methodology established by Williamson and Kligman [17]. Briefly, a sterile 2.5 cm diameter ring of PVC tubing (Nalgene® Labware, Rochester, NY) was placed over the skin lesion of patient, then, 1 ml sterile rinse solution (0.069M Na_2HPO_4, 0.0064M NaH_2PO_4, and 0.1% Tx-100) was administered inside the ring chamber that was held tightly on the skin to prevent leakage. The rinse solution was stirred around in the chamber with a sterile Teflon® rod (Scientific Commodities Inc., Lake Havasu City, AZ) for 15-20 times and collected. This collection was repeated and 2 ml total rinse solution was obtained. This methodology has been shown to be 95% quantitative for aerobic surface bacteria [17]. The wash solution was then aliquotted for immunoblotting analysis of LTA, and bacterial quantitation. *S. aureus* and other bacteria including *S. epidermidis* (coagulase-negative staphylococcus) colonies were quantified in the microbiology lab in Indiana University Hospital by limiting dilution assay.

Measurement of LTA in wash fluid specimens

Quantitative measurements of LTA protein were as previously described [9,13]. Briefly, 32 μl rinse solution from each patient sample was separated on 18% Tris-HCl gradient gel (Bio-Rad Laboratories, Hercules, CA), along with standards of 10, 5, 2.5, and 1 ng LTA protein (Sigma-Aldrich, St. Louis, MO) dissolved in same rinse solution loaded on the same electrophoresis gel. LTA protein was determined by immunoblotting with LTA monoclonal antibody (QED Bioscience, San Diego, CA) and enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). The arbitrary optical densities were measured by Image J Software (NIH, Bethesda, MD). The quantification of LTA was determined according to the standard curve drawn. We estimate that the limit of detection of LTA in wash fluid in this assay is approximately 7 ng/ml.

ELISA

1.7% or 10% (v/v) of wash fluid from AD patient samples were incubated for 48 hr in the DC culture medium. Cytokine concentrations in supernatants were detected by ELISA using commercially available antibodies from eBiosciences.

Intracellular Calcium Mobilization Studies

Intracellular calcium mobilization response following the PAF-R agonist CPAF was used to assess DC for the presence of functional PAF-Rs. As previously described [9], DC were preloaded with the calcium-sensitive indicator FURA-2AM (3μM in HBSS; Molecular Probes, Eugene, OR) at 37 °C for 90 min, followed by washing, re-suspending and maintained in HBSS at room temperature before use. FURA-2-AM fluorescence was monitored in a Hitachi F-4010 spectrophotometer with excitation and emission wavelengths of 331 and 410 nm, respectively. Intracellular calcium levels were calculated as previously described [9].

Statistical Analysis

Statistical significance between groups was determined using a nonparametric t-test. Differences were considered significant when $p < 0.05$ (*).

Results

Wash fluid from AD lesions induces cytokine production in DC

Bacterial infection is a known trigger for worsening of AD. To assess whether infected AD skin lesions contain agents that can activate DC cells, we tested the ability of wash fluid obtained by quantitative culture technique from children with inflamed and potentially infected AD lesions. As shown in Table I, ten samples derived from eight patients were used in these studies. Two of the subjects (A-2, B-2) represent follow-up visits 14 days following treatment with topical steroids and oral antibiotic, cephalexin. Of the ten samples, six contained *S. aureus* indicative of secondary infection. Also, six of the samples contained other strains of bacteria and four contained detectible levels of LTA. We then prepared BMDC and cultured them with low levels (final concentration 1.7% v/v) of patient wash fluid samples followed by ELISA to measure the amounts of cytokines produced by DC. We first assessed the pro-inflammatory cytokine IL-6. Seven of the ten samples resulted in IL-6 production that was above the amount from untreated (UT) cells (Fig. 1A). An increase in concentration to the final concentration 10% v/v of patient wash fluids resulted in significant increases in IL-6 production suggesting the dose-dependent activation by a soluble factor in AD lesion wash fluid (Fig. 1A).

Samples from E1, F-1 and H-1 did not produce meaningful levels of IL-6 even at the high concentration of 10% suggesting that the three lacked the component inducing cytokine production in DC. Samples from A-2 and B-2 induced less DC cytokine response than their counterparts, A-1 and B-1, suggesting that the two-week treatment regimen including antibiotic course was capable of removing the cytokine inducing factor(s) from the two patients' wash fluids. Although 7 patients' samples were able to activate IL-6 production only one patient wash fluid (A-1) was capable of inducing IL-1β and TNFα production (Fig. 1B). All patients' samples produced anti-inflammatory IL-10 in various amounts. High IL-10 producers such as B-1 and C-1 also secreted more IL-6 than the rest suggesting a correlation in the production of these two cytokines.

In regards to the potential factors, it was noted in the small number of specimens that the amount of LTA seems to correlate strongly with the potential to induce DC activation. Patient sample A-1 contains almost three-fold higher LTA than any other sample and was the only specimen able to activate other pro-inflammatory responses, IL-1β and TNFα. Together, these data suggest that AD lesions contain soluble factor(s) with biological activity capable of activating DC to produce cytokines.

Wash fluid-induced DC cytokine production involves MyD88

The lipoprotein LTA is a known inducer of DC cytokine production [18-20]. Since all wash fluids containing measurable amounts of LTA had the ability to stimulate DC cytokine production, we asked if LTA in wash fluid is responsible for the biological activity. Because LTA is a potent TLR2 agonist, and TLR2 requires the MyD88 adapter protein for signaling [7], we tested whether MyD88 participates in LTA-mediated activation of DC. To do this, we prepared BMDC from wild type and MyD88-deficient mice (*Myd88-/-*), cultured BMDC in the presence of wash fluids, and then measured the amount of cytokines present in the supernatant. For this experiment, we used wash fluid from two high-responders (A-1, C-1) and two low responders (A-2, H-1). Figure 2A illustrates that the cytokine production was abolished in *Myd88-/-* (Fig. 2A). As expected, treating BMDC with LPS or LTA, both of which depend on MyD88, showed compromised cytokine production in the absence of MyD88.

In addition to LTA, lipopolysaccharide (LPS) is a potent activator of DC, which also requires MyD88. Wash fluid contains bacterial products including possibly LPS and therefore it is possible that the results seen with the deficiency of MyD88 could be a compound effect due to the lack of both LTA- and LPS-mediated signaling. To test this, we employed Polymyxin B, an antibiotic capable of binding LPS components [21], to inhibit a potential effect of LPS in wash fluids. We treated wash fluid with Polymyxin B prior to adding them to the DC culture. The results showed <u>a significant</u> difference in IL-6 production (Fig. 2B). Together, the data suggest that LTA and to a lesser extent LPS in patient samples are the primary components contributing to the biological activity of wash fluid which activates DC cytokine production in a MyD88-dependent manner.

PAF-R in DC is functional

LTA can also act as an agonist for the PAF-R in several model systems [8,9]. Because LTA levels correlate with the potency of wash fluid to activate DC, we examined whether LTA exerts its effect through the PAF-R in DC. Although human DC have been reported to respond to PAF-R agonists [22-24] it is necessary to test whether the PAF-R is expressed and functional in murine DC. To do this, we loaded BMDC with FURA-2AM, treated with the PAF-R agonist carbamoyl PAF (CPAF), and measured the response by the intracellular calcium mobilization. DC responded to CPAF, confirming that murine DC express functional PAF-R (Fig. 3). The CPAF triggered response was blocked by pretreatment with 10 μM of the PAF-R antagonist WEB2086. These studies indicate that BMDC express functional PAF-Rs.

PAF-R does not play a role for cytokine production by DC

Having observed the presence of functional PAF-R in DC and because triggering PAF-R in other cell types induces the production of IL-6 and TNF-α [25-27], we next investigated whether PAF-R is involved in cytokine production. In particular, we designed these experiments to evaluate whether the DC PAF-R alone could stimulate cytokine production, or could prime DC for exaggerated cytokine production, or exerted an inhibitory effect. To do this, we first examined the ability of DC to produce cytokines in response to CPAF by treating DC with LPS alone or together with CPAF. Adding CPAF did not result in an increased IL-6 compared to that of LPS without CPAF (Fig. 4A). Similarly, CPAF treatment of DC did not result in the production of TNF-α, nor was TNF-α production augmented by co-treatment of CPAF and LPS in wild-type DC (*data not shown*). Together, the data suggest that CPAF does not augment LPS responses.

To further examine the role of PAF-R in DC activation and cytokine production, we tested DC lacking PAF-R. As seen with the wild type cells in Figure 3, *Paf-r-/-* cells showed little

difference in IL-6 levels regardless of CPAF addition (Fig. 4A). Next, we treated wild-type and *Paf-r*^{-/-} DC with various concentrations of LTA and found that the amount of IL-6 was comparable between the two (Fig. 4B). In contrast, the absence of MyD88 severely reduced IL-6 production, yet at high concentrations *Myd88*^{-/-} cells were still capable of producing small amounts of IL-6 (Fig. 4B). Production of cytokines IL-1β, TNFα, and IL-10 showed similar patterns as with IL-6 (*data not shown*). Because DC rely on MyD88 to produce cytokines upon treatment with wash fluid we asked whether the PAF-R signaling pathway is also involved in making cytokines. When wild-type and PAF-R^{-/-} DC were treated with wash fluid samples from four patients, both showed equivalent IL-6 (Fig. 4C). Together, these data demonstrate that TLR pathways, but not PAF-R play a role in DC cytokine production induced by LTA or wash fluid samples from infected AD lesions.

Discussion

Bacterial infection with *S. aureus* is a known trigger for worsening of AD [1-4]. The demonstration that active AD lesions have decreased levels of antimicrobial peptides in comparison to normal skin or other inflammatory diseases such as psoriasis has provided a mechanism for the increased incidence of staphylococcal infections in this population [28]. The exact mechanism(s) by which staphylococcal impetiginization can worsen AD is unknown at present. Though the keratinocyte has been implicated in this process, other immune cells such as DC could certainly be participants. The present studies demonstrate that wash fluid derived from a small group of clinically infected AD lesions possessed soluble factors capable of activating murine DC. It has been previously demonstrated that high levels of these same cytokines, especially IL-6, are found in wash fluid samples derived from infected AD lesions [13].

The exact soluble factor(s) found in the skin lesions which trigger DC cytokine production remain somewhat unknown. However, our current study provides several lines of evidence implicating LTA as a major player in this process. Wash fluid samples that activated DC cytokine production also contained measureable amounts of LTA. The use of *MyD88*^{-/-} DC confirmed that the activation of DC is TLR-dependent. Inasmuch as inhibition of LPS with polymixin decreased the biological activity of the patient samples, endotoxin is also playing a role in the biological activity of the wash fluid samples. There have been several studies examining the role of TLR2 in AD, though questions remain [4]. Some groups have linked TLR2 to recurring bacterial infections, similar to that of *S. aureus* in AD [29]. Since treatment of human DC with LTA also results in cytokine production [30,31] the present study lends support to the role TLR2 in the worsening of AD through LTA-induced activation of pro-inflammatory cytokines from DC.

The present studies confirm that DC express functional PAF-R. The role of the DC PAF-R is unclear, though it has been reported that PAF or oxidized high density lipoproteins that exert PAF-R agonistic activity inhibit the migration of DC [32]. Though LTA has been shown to act as an agonist of the PAF-R [8,9], the present studies demonstrate that PAF-R activation does not induce appreciable cytokine production from DC. Moreover, PAF-R deficiency had little impact on the ability of DC to produce cytokines in response to LTA and AD wash fluid. Interestingly, however, PAF-R ligation induced Ca++ mobilization indicating that the DC PAF-R is functional. Nonetheless, neither PAF-R-mediated signaling nor intracellular Ca++ signaling alone is sufficient for DC to produce cytokines. Moreover, the DC PAF-R does not exert augmentative or inhibitory effects [33] on cytokine production induced by LTA or LPS.

The present studies suggest that DC could be involved in the worsening of AD associated with *S. aureus* infection. Evaluation of wash fluid samples using quantitative bacterial

culture methodology from clinically impetiginized AD lesions revealed that relatively small amounts of the wash fluid (even 1.7% v/v) was found to exert potent cytokine producing effects on murine DC. We propose that staphylococcal products such as LTA are involved in this process. Further studies are necessary to elicit other soluble factors in AD lesions and their respective effects on the pathogenesis of AD in humans.

Acknowledgments

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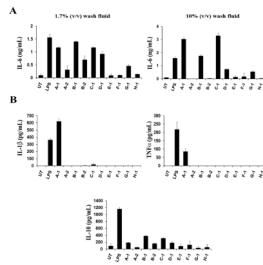
Fig. 1. Wash fluid from infected AD lesions stimulates cytokine production in DC
Murine DC from C57BL/6 mice were treated with LPS (100 ng/ml) or wash fluid derived
from infected AD lesions of subjects in Table I. UT; untreated. Supernatants were collected
after 48 hours and the amounts of indicated cytokines were measured by ELISA. (A) DC
were cultured at two different concentrations of wash fluid. 17μl of patient samples was
added to 1ml of culture medium (1.7% (v/v)) or 100μl of patient samples was added to 1ml
(10% (v/v)). IL-6 cytokine production was quantified from both. (B) IL-1β, TNF-α, and
IL-10 cytokine production was quantified for patient samples cultured at 10% (v/v). The
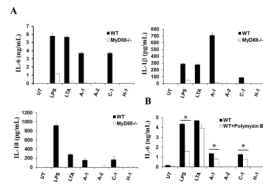data represent mean +/- SD from triplicate samples.

Fig. 2. Wash fluid-mediated cytokine production is MyD88-dependent

Murine DC from C57BL/6 (WT) or *Myd88*-/- mice were treated with LPS (100 ng/ml), LTA (10 μg/ml), or wash fluid derived from infected AD lesions of subjects in Table I (final concentration 10 % v/v). UT; untreated. Supernatants were collected after 48 hours and analyzed by ELISA. (A) IL-6, IL-1β, and IL-10 production was quantified from two high-responders (A-1, C-1) and two low responders (A-2, H-1). (B) Wash fluids (final volume 10% (v/v)), LPS (100ng), or LTA (10μg) were pre-incubated with Polymyxin B (2.5μg) for 15 minutes at room temperature and then added to the DC culture. Samples not treated with Polymyxin B were cultured in the same way. The data represent mean +/- SD from triplicate samples.

Fig 3. Murine DC have functional PAF-R
Murine DC from C57BL/6 mice were loaded with calcium sensitive fluorescent dye FURA-2AM, and treated with 100 nM of the PAF-R agonist CPAF, and B. 10 μM of the PAF-R antagonist WEB2086 (WEB) prior to 100nM CPAF. Intracellular calcium levels were measured over time. These data are representative of at least three separate experiments.
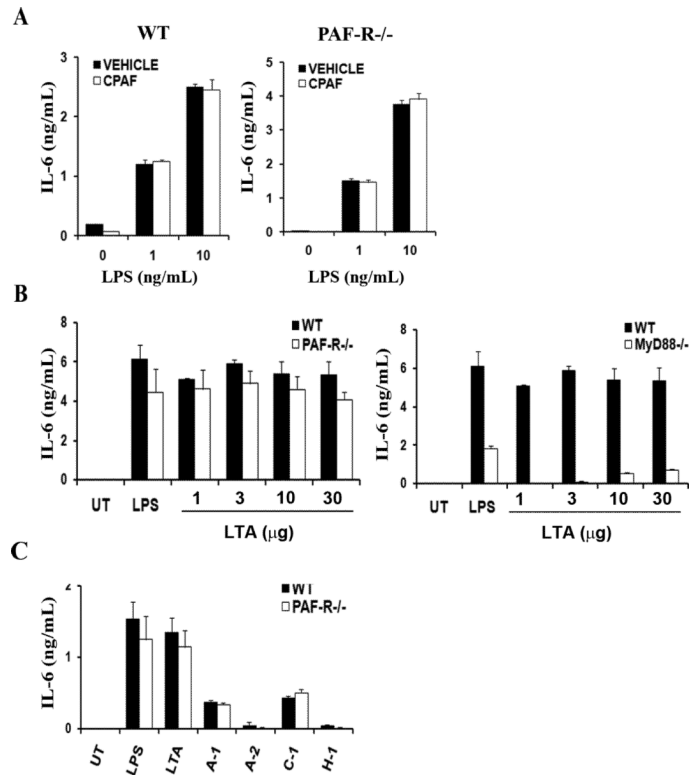
Fig. 4. PAF-R mediated signaling does not play a role in cytokine production by DC
(A) Murine DC from C57BL/6 or *Paf-r-/-* mice were treated with the PAF-R agonist CPAF
(100nM) alone, or as co-activation with 1 or 10 ng/ml of LPS. Forty-eight hours later, the
amount of IL-6 in the supernatant was measured by ELISA. The data represent mean +/- SD
from triplicate samples. (B) Murine DC from PAF-R-/- or MyD88-/- mice together with
C57BL/6 mice were treated with LPS (100 ng/ml) various doses of LTA (1-30 μg/ml), or
left untreated. IL-6 was measured as in (A). (C) Murine DC from C57BL/6 or PAF-R-/-
mice were treated with LPS (100ng/ml), LTA (10μg/ml), or patient wash fluids (final
volume 10% (v/v)). Forty-eight hours later, IL-6 was measured in supernatants by ELISA.
The data represent mean +/- SD from triplicate samples.

Table I

Comparison of clinical assessment of inflammation, amounts of *S. aureus*, and levels of LTA in AD lesions

The visit number, EASI score of the tested lesion, concentration of *S. aureus* or other bacteria (in CFU/ml), and LTA levels are listed for the 10 wash fluid specimens derived from 8 subjects.

Sample #	Visit	EASI-les	Log SA (CFU/ml)	Other bacteria (log CFU/ml)	LTA (ng/ml)
A-1	1	8	6.5	None	236
A-2	2	3	2.0	None	ND
B-1	1	12	5.6	*S. epi* (4.1)	16
B-2	2	6	ND	*S. epi* (2.8); *Strep viridans* (3.3)	ND
C-1	1	9	5.6	*S. epi* (3.2)	18
D-1	1	9	5.9	None	83
E-1	1	10	7.0	None	ND
F-1	1	7	ND	*S. epi* (2.8)	ND
G-1	1	7	ND	*S. epi* (3.4)	ND
H-1	1	9	ND	*S. epi* (2.6); *Pseudomonas aeruginosa* (2.3)	ND