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Infected Atopic Dermatitis Lesions Contain Pharmacologic Amounts of Lipoteichoic Acid

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Abstract

Background—Bacterial infection with *Staphylococcus aureus* is a known trigger for worsening of atopic dermatitis (AD); the exact mechanisms by which bacterial infection worsens dermatitis are unknown.

Objective—We sought to characterize the amounts of the biologically active bacterial lipoprotein lipoteichoic acid (LTA) in infected AD lesions.

Methods—Eighty-nine children with clinically impetiginized lesions of AD were enrolled in this study. A lesion was graded clinically using the Eczema Area and Severity Index (EASI), and then wash fluid obtained from the lesion for quantitative bacterial culture, and measurement of LTA and cytokines. The staphylococcal isolate was tested for antibiotic susceptibilities. The patients were treated with a regimen that included topical corticosteroids and systemic antibiotics and the lesion was re-analyzed after two weeks.

Results—*S. aureus* was identified in 79 of 89 children enrolled in the study. The bacterial CFU correlated with the EASI lesional score ($p = 0.04$). LTA levels up to 9.8 $\mu\text{g/ml}$ were measured in the wash fluid samples and the amounts correlated with the lesional EASI scores ($p = 0.01$) and *S. aureus* CFU ($p < 0.001$). Approximately 30% of clinically impetiginized AD lesions contained greater than 1 $\mu\text{g/ml}$ LTA, amounts that exert effects on various cell types *in vitro*. Moreover,

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injection of skin tissue *ex vivo* with amounts of LTA found in AD lesions resulted in epidermal cytokine gene expression.

Conclusions—Pharmacologic levels of LTA are found in many infected atopic dermatitis lesions.

Clinical Implications—These findings suggest that staphylococcal LTA could be an important mediator of the increased skin inflammation associated with infected AD.

Capsule Summary—These studies demonstrate high levels of staphylococcal LTA are found on impetiginized AD lesions. Moreover, subjects harboring MRSA exhibited greater total body involvement of AD.

Keywords

Lipoteichoic acid; *Staphylococcus aureus*; atopic dermatitis; Tumor necrosis factor-alpha; interleukin-8

Introduction

Staphylococcus aureus infections are known triggers for skin inflammation and can modulate immune responses. These effects can be due to either direct invasion by the bacteria or by bacterial products. Patients with atopic dermatitis (AD) are particularly sensitive to staphylococcal skin infections, which are known to worsen their skin disease (1–3). The mechanisms by which staphylococcal bacteria can worsen AD are active areas of study (4,5). Possible mechanisms include cytokine production following direct infection of keratinocytes or immune cells (6,7). Toxins encoded by staphylococcal bacteria that act as superantigens have also been implicated in the worsening of AD in response to infection (reviewed in 8). In addition, other toxins including the pore-forming protein alpha toxin (alpha-hemolysin) induce cytokines and stimulate inflammatory reactions (9,10).

Potential soluble 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) (11) as well as the platelet-activating factor receptor (PAF-R) (12,13). Since LTA has not been previously quantitated *in vivo*, the objective of the present studies was to quantitatively assess the staphylococcal CFU, the amounts of LTA and cytokines present in clinically infected AD lesions in children and compare this with the amount of skin inflammation. We demonstrate that amounts of LTA correlate with patient EASI score, suggesting that LTA may be an important component of the ability of *S. aureus* to exacerbate AD lesions.

Methods

Reagents

All chemicals were obtained from Sigma unless indicated otherwise.

Atopic Dermatitis Subjects

Subjects with AD diagnosed using criteria of Hanifin and Rajka (14) were enrolled in these studies approved by the Indiana University Institutional Review Committee. Subjects were not exposed to oral antibiotics for a period of one month before the study. Subjects enrolled into the study underwent a clinical assessment of a clinically-infected lesion of dermatitis using the Eczema Area and Severity Index (EASI) (15), as well as an EASI scoring of entire body. Wash fluid was removed from the lesion and the subject treated with appropriate therapy, to include oral cephalexin antibiotic (or clindamycin if allergic to cephalosporins or penicillins). 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 (13) using the methodology established by Williamson and Kligman (16). 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 Na₂HPO₄, 0.0064M NaH₂PO₄, 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 (16). The wash solution was then aliquotted for immunoblotting analysis of LTA, and bacterial quantitation. *S. aureus* and *S. epidermidis* (coagulase-negative staphylococcus) colonies were quantified in the microbiology lab in Indiana University Hospital by limiting dilution assay, and antibiotic susceptibilities assessed by standard methodology.

Measurement of LTA in wash fluid specimens

Quantitative measurements of LTA protein were as previously described (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. LTA was quantified based upon area (ng/cm²) of the chamber that then was converted to volume (ng/cm³) based upon estimation of 0.1 cm effective epidermal thickness.

Cytokine multiplex analysis of wash fluid specimens

Patient wash fluids were collected and levels of IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, IFN-γ and TNF-α were measured using the Multiplex Bead Immunoassays as per manufacturer's protocol (Millipore, Billerica, MA). A Luminex 200 instrument (Luminex Corporation, Austin, TX) was used for data acquisition and analysis. Cytokine concentrations were calculated using Bio-Plex Manager 2.3 software with a five parameter curve-fitting algorithm applied for standard curve calculations. Cytokines in wash fluid were quantified based upon area (ng/cm²) of the chamber that then was converted to volume (ng/cm³) based upon estimation of 0.1 cm effective epidermal thickness.

Measurement of cytokines in human skin explants

Human skin from abdominoplasty surgeries was obtained, the fat trimmed, washed multiple times in PBS, and cut into 2 × 2 cm sections. The tissue was placed on 10 cm dishes with a small amount of HBSS and allowed to incubate at 37°C. The skin was injected intradermally with 1 or 10 µg of LTA or 100 µl saline using a 30 gauge needle underneath the epidermis to produce a wheal. The tissue was then incubated for 4 or 24h in incubator at 37°C. Skin was harvested by freezing the epidermis with liquid nitrogen to allow epidermal removal with a 5 mm curette. We have used histological sections following this treatment to confirm that this technique selectively removes epidermis. Skin tissue was homogenized and total RNA was isolated using RNeasy Mini Kit (Qiagen) as described by the manufacturer. cDNA were synthesized with ReactionReady First-Strand cDNA synthesis kit (SuperArray, Frederick, MD) and qRT-PCR was performed by the comparative threshold cycle (ΔC_T) method and normalized to GAPDH (17). The primers used for human TNF-α, IL-6, IL-8 and GAPDH were from SuperArray.

Statistical analysis

For all correlation analyses, Spearman rank correlation coefficient was used. Wilcoxon rank sum test was applied to compare EASI scores and LTA levels between oxacillin-resistant and oxacillin-sensitive staphylococcus groups. Wilcoxon signed rank test was used to detect a significant change in outcomes of interest at visit 2 from visit 1.

Results

Correlation between lesional concentration of *S. aureus* bacteria and clinical inflammation

Eighty-nine children (ages 3 months-6 years) with AD were enrolled in studies to clinically define eczema severity and quantitatively or qualitatively determine the levels of lesional bacterial products before and after treatment. The experimental procedure, including a photograph of a typical patient at first and second visits is depicted in Fig 1. Lesion wash fluid was obtained and used for quantitative *S. aureus* culture or aliquotted and stored at -80°C before it was used for measurement of other proteins/cytokines. An isolate of the *S. aureus* was assessed for antibiotic sensitivities. Of the 89 subjects, 64 returned for their second visit two weeks later. The lesions varied amongst the subjects, with 72 lesions from extremities, 12 from trunk/back/abdomen, and five from the face. No significant differences were seen in staphylococcal CFU nor lesional EASI scores amongst samples taken from various locations (*data not shown*). Treatment resulted in improvement of the lesional EASI scores from (mean $\pm$ SD) 9.8 ± 1.8 at visit one to 4.4 ± 1.9 at visit two ($p < 0.0001$). Of the initial visits, *S. aureus* was measured in clinically impetiginized atopic dermatitis lesions in all but ten subjects. Three of these *S. aureus*-negative lesions expressed low levels ($< 10^3$ CFU/ml) of group A streptococcus, and two others expressed low levels ($< 10^3$ CFU/ml) of coagulase-negative staphylococcus. Previous studies (18–21) have reported that the amount of skin inflammation tends to correlate with the numbers of *S. aureus* bacteria, with levels above 10^6 CFU/ml indicative of clinical infection. Again, treatment resulted in decreased amounts of *S. aureus* (log CFU/ml) on the lesional skin samples from 5.5 ± 2.0 at visit one to 1.7 ± 2.3 at visit two ($p < 0.0001$). As shown in Fig 2A, plotting the clinical EASI score of the lesions versus the concentration of *S. aureus* bacteria in the wash fluid for all of the visits (both first and second) indicated that the amount of bacteria positively correlated with the lesional clinical score (Spearman Correlation coefficients visit 1: $r = 0.21$, $p = 0.04$; visit 2: $r = 0.35$, $p = 0.005$) and total EASI clinical score (Spearman Correlation coefficients visit 1: $r = 0.23$, $p = 0.03$; visit 2: $r = 0.45$, $p = 0.0003$). Though coagulase-negative staphylococcal species were also detected in some wash fluid samples (either along with *S. aureus* or by themselves), the amounts were always less than 10^3 CFU/ml. Moreover, the levels of coagulase-negative staphylococcal species did not correlate with the clinical scoring of the individual lesion (*data not shown*).

Significant levels of LTA are found in infected AD lesions

The gram-positive bacterial lipoprotein LTA has immunodulatory effects through its ability to stimulate TLR2 and PAF-Rs (11–13). The next studies examined the amounts of LTA in the wash fluid derived from the skin lesions. Fig 2B depicts the levels of LTA versus *S. aureus* CFU for all 153 specimens. There was a strong positive correlation between amounts of LTA and staphylococcal bacteria (Spearman Correlation coefficients visit 1: $r = 0.66$, $p < 0.001$; visit 2: $r = 0.53$, $p < 0.001$). Measureable amounts of LTA were only found in one sample lesion where bacterial counts were below 10^4 CFU/ml. Above bacterial counts of 10^6 CFU/ml, 49 of 53 specimens (92%) contained measureable amounts of LTA. Yet below bacterial counts of 10^6 CFU/ml, only 14 of 99 specimens (14%) contained measureable levels of LTA. As shown in Fig 3A, the levels of LTA also positively correlated with the lesional EASI score (Spearman Correlation coefficients visit 1: $r = 0.27$, $p = 0.01$; visit 2: $r = 0.28$, $p = 0.02$) and total EASI score (Spearman Correlation coefficients visit 1: $r = 0.28$, $p = 0.009$; visit 2: $r = 0.38$, $p = 0.002$). It should be noted that the concentration of LTA needed to induce responses

in vitro is approximately 1 µg/ml (11–13,22,23). Examination of levels of LTA found in first visits indicated that 24 of 79 lesions that grew *S. aureus* (30%) contained greater than 1 µg/cm³ of this bioactive lipoprotein (Fig 3B). These studies demonstrate that “pharmacological” amounts of LTA can be found in impetiginized AD lesions.

Staphylococcal antibiotic sensitivities do not correlate with lesional EASI scores or levels of LTA or IL-8, but correlate with total EASI scores in children with infected AD

The next studies examined whether or not a correlation existed between the antibiotic susceptibilities of the *S. aureus* and the clinical level of inflammation in our subjects. Methicillin-resistant *S. aureus* strains (MRSA) are becoming more common in children with atopic dermatitis and it has been suggested that these patients are a reservoir for resistant staphylococcus species in the community (24). Yet it is not known whether the antibiotic sensitivity of *S. aureus* strains impacts the amount of inflammation in AD patients. As shown in Table I, in 19 of a total of 79 lesions (24%) the *S. aureus* isolates were resistant to the antibiotic oxacillin. The EASI score of the lesions which contained a MRSA were not statistically different from the lesions containing a methicillin-sensitive staphylococcus (MSSA) species (mean ± S.E.: 9.9 ± 0.2 versus 10.2 ± 0.4, *p*=0.66). However, the total body EASI score of subjects from which an MRSA was found was significantly greater than in those subjects harboring an oxacillin-sensitive species (614 ± 78 versus 377 ± 28; *p* <0.01). Consistent with the lack of differences in inflammation between lesions containing oxacillin-sensitive versus –resistant *S. aureus* species, the levels of LTA measured in the lesions were not different (Table I). Moreover, the amounts of the proinflammatory cytokine IL-8 was not different in wash fluid samples obtained from lesions harboring MRSA versus MSSA (Table I). These studies indicate that the staphylococcal antibiotic sensitivity did not affect the inflammatory characteristics of the individual lesion tested yet were associated with greater total body AD.

Cytokine analysis of wash fluid specimens from impetiginized AD lesions

To define the level and type of inflammatory responses in the lesions, we next determined the presence of cytokines in the wash fluid. As outlined in *Methods*, wash fluid was subjected to multiplex analysis to measure IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, IFN-γ and TNF-α proteins. As shown in Table II for visit 1, levels of all of the cytokines except IL-12 and IFN-γ were measured in clinically impetiginized atopic dermatitis lesions. The levels of the inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α were positively correlated with LTA concentrations for the first (see Table II and Supplemental Fig. S-1) and second visits (see Supplemental Table S-1 and Fig. S-1). Of interest, measureable levels of the TH2 cytokine IL-4 were also found in many of the wash fluid samples, yet IL-4 concentrations did not correlate with LTA levels (Table II and Supplemental Fig S-1). Though there was a positive association between the concentration of inflammatory cytokines and LTA, it should be noted that there were no associations between cytokine levels and *S. aureus* oxacillin susceptibility (*data not shown*). These studies indicate that biologically relevant levels of pro-inflammatory (IL-1β, IL-6, IL-8, TNF-α) and TH2 cytokines IL-4 and IL-13 can be measured in many wash fluid specimens derived from clinically impetiginized AD lesions.

Intradermal injection of LTA results in epidermal cytokine production in human skin explants

The correlation between the levels of inflammatory cytokines and LTA concentrations lead us to hypothesize that direct administration of LTA to human skin would induce the expression of inflammatory cytokines. Skin tissue from abdominoplasty specimens was treated by intradermal injection of 1, or 10 µg LTA or PBS vehicle right below the epidermis using a 30 gauge needle. At 4 and 24 h the epidermis was harvested from the skin explant and epidermal cytokine mRNA measured. As demonstrated in Fig. 5, LTA treatment resulted in an up-

regulation of epidermal *TNF*, *IL-6* and *IL-8* gene expression. These studies indicate that levels of this bacterial lipoprotein that are present in infected AD lesions can stimulate cytokine gene expression in human epidermal skin.

Discussion

Bacterial infection with *S. aureus* is a known trigger for worsening of AD (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 (25).

The present studies confirm previous reports (18–21) of an association between high amounts of staphylococcal bacteria and clinical worsening of AD lesions. There was not an association between antibiotic susceptibility pattern (eg, methacillin-sensitive or –resistant) and bacterial levels, IL-8 protein levels, nor clinical parameters of the individual infected atopic dermatitis lesions tested in this study (Table I). However, subjects harboring MRSA had greater total body clinical dermatitis scores. These findings could have clinical importance and the presence of widespread impetiginized AD should alert the clinician to consider the possibility of infection with MRSA.

Bacterial products including the cell wall lipoprotein LTA could be involved in staphylococcal-mediated skin inflammation. LTA has been shown to act on TLR2, as well as exerting agonistic effects on the PAF-R (11–13). Of note, recent studies from our group has shown that LTA can trigger IL-10 production in murine skin, and also inhibit contact hypersensitivity to the chemical dinitrofluorobenzene (a TH1 process) via its ability to act as a PAF-R agonist (13).

LTA has been shown to exert other immunological effects that could implicate it in worsening of atopic dermatitis. Peptidoglycan and LTA both have been shown to stimulate IL-5 production in peripheral blood mononuclear cells derived from patients with AD, yet not in control subjects (26). In mice, application of topical LTA along with dust mite antigen resulted in enhanced mite IgE antibody production (27). Finally, topical application of LTA to mice that have been percutaneously sensitized to allergens has been reported to result in a dermatitis that has features of AD (28).

The levels of LTA necessary to exert the above effects, and also to act upon TLR2 or PAF-R appear to be in the microgram/ml range. This is in contrast to the gram-negative bacterial product lipopolysaccharide, which exerts effects at 100–1000 × lower concentrations (29). The present studies indicate that levels of LTA which are pharmacologically active (i.e, above 1 microgram/cm³) are commonly encountered (30% of first visits) in subjects with impetiginized atopic dermatitis lesions. Though LTA was measured in the majority of visit 1 specimens (57 of 89), a number of specimens did not contain this lipoprotein. Possible reasons for the lack of LTA in wash fluid specimens could include that the lesion tested had been disturbed (eg, washed) before being tested. It should also be noted that the limits of detection of our immunoblot-based assay is estimated to be about 25 ng/cm³, suggesting the possibility that some of the negative specimens contained small amounts of LTA.

TLR2, a major target for LTA, has been implicated in host immunity against *S. aureus* (30). Indeed, studies using mice deficient in TLR2 demonstrated a greater susceptibility to intradermal or subcutaneous staphylococcal infections (31). A single nucleotide polymorphism (SNP) in the *TLR2* gene (R753Q), which apparently suppresses cytokine activation has been reported (32). Humans heterozygous for this TLR2 mutation have been reported to be more prone to staphylococcal infections and have increased incidence and severity of AD (33,34). These studies and our finding that intradermal injection of microgram amounts of LTA can stimulate cytokine production in clinically normal skin explants all fit with the notion that

bacterial cell wall products such as LTA could be important pathogenic factors for the worsening of AD seen with *S. aureus* infection.

In summary, the present studies confirm the importance of *S. aureus* as a potent trigger for worsening of AD. Evaluation of wash fluid samples using quantitative bacterial culture methodology from clinically impetiginized AD lesions revealed microgram amounts of LTA, quantities that are sufficient to induce inflammatory cytokine gene expression in epidermal cells, suggesting that this product could be an important mediator in worsening of AD. These studies indicate that this bacterial-derived lipoprotein warrants further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

AD	Atopic dermatitis
CFU	colony forming unit
LTA	Lipoteichoic acid
PAF	Platelet-activating factor
TLR	Toll-like receptor
IL	interleukin
TNF	Tumor necrosis factor
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin-sensitive <i>Staphylococcus aureus</i>

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Fig. 1. Outline of Research Protocol

1A. Outline of clinical protocol involving wash fluid derived from clinically impetiginized AD lesions. **1B.** Typical subject with clinically impetiginized AD lesion at first visit, and second visit two weeks later following treatment with topical corticosteroid and oral antibiotic.

Fig. 2. Amounts of *S. aureus* Bacteria Versus Clinical Assessment of Inflammation and LTA levels in AD Subjects

Wash fluid obtained from 89 subjects at first visits (red circle) and 64 at two-week follow-up visits (blue triangle) were assessed for amounts of *S. aureus* bacteria and this was compared to **2A. Lesional EASI scores**. Spearman Correlation coefficients visit 1: $r = 0.21$, $p = 0.04$; visit 2: $r = 0.35$, $p = 0.005$ and **2B. LTA levels**. Spearman Correlation coefficients visit 1: $r = 0.66$, $p < 0.001$; visit 2: $r = 0.53$, $p < 0.001$.

Fig. 3. Amounts of LTA Versus Clinical Assessment of Inflammation in AD Subjects

3A. Wash fluid obtained from 89 subjects at first visits (red circle) and 64 at two-week follow-up visits (blue triangle) were assessed for amounts of LTA and this was compared to lesional EASI scores. Spearman Correlation coefficients visit 1: $r = 0.27$, $p = 0.01$; visit 2: $r = .28$, $p = 0.02$ **3B.** Wash fluid obtained from 79 subjects at first visits which were positive for *S. aureus* were assessed for amounts of LTA and this was compared to lesional EASI scores. The line depicts a concentration of LTA of $1 \mu\text{g}/\text{cm}^3$.

Fig. 4. Effect of Intradermal LTA Injections on Epidermal Cytokine mRNA Levels in Human Skin Human skin explants were treated with intradermal injections of 1 or 10 μ g LTA, or PBS vehicle. Four or 24 h later the epidermis was removed and *TNF*, *IL-6* and *IL-8* mRNA measured and normalized to *GAPDH* mRNA levels. The data depicted are the mean $\pm$ SD normalized cytokine mRNA of LTA-treated skin relative to PBS control from triplicate samples of a representative experiment (from three). * denotes statistically significant ($p < 0.05$) differences from PBS control-injected skin.

Table 1
Comparison of Antibiotic Susceptibilities, Clinical Assessment of Inflammation, Amounts of *S. aureus*, and Levels of LTA and IL-8 in Staphylococcal-positive AD Lesions at Visit 1

The EASI score of the tested lesion and total body EASI scores, concentration of *S. aureus* bacteria (in CFU/ml) and LTA and IL-8 levels were compared in samples which the bacterial isolate was sensitive (MSSA) or resistant (MRSA) to the antibiotic oxacillin.

Antibiotic Susceptibility (# specimens)	EASI lesional	EASI total	Log [<i>S. aureus</i>]	[LTA] (ng/cm ³)	[IL-8] (pg/cm ³)
MSSA (60)	9.9 (1.7)	377 (220)	6.1 (1.3)	954 (1758)	10760 (15452)
MRSA (19)	10.2 (0.4)	614 (339) *	6.5 (0.9)	868 (1572)	6956 (8664)

Each of the above are the mean ± SE.

* denotes that the EASI total in MRSA specimens was significantly (p = 0.003) greater than EASI total in MSSA specimens.

Table II
Cytokine Concentrations in Clinically Infected AD Lesions

The above cytokines (in pg/cm³) were measured in 89 first-visit wash fluid specimens by multiplex analysis. The amounts of various cytokines were compared to LTA levels, and listed as Spearman Correlation Coefficients. Cytokines are grouped by roles in general inflammation (IL-1 β , TNF- α , IL-6, IL-8, IL-17), TH2 immunity (IL-4, IL-5, IL-10, IL-13) or TH1 immunity (IL-12, IFN- γ).

Cytokine	Mean	Median	Skewness	Std Dev	Minimum	Maximum	Correlation with [LTA]
IL-1 β	242	36	7.7	1046	1.1	9231	$r = 0.38$ $p = 0.0002$
TNF- α	50	30	2.8	59	0	360	$r = 0.40$ $p < 0.0001$
IL-6	366	128	2.5	571	0	2831	$r = 0.42$ $p < 0.0001$
IL-8	9422	4598	2.8	13,696	22	83,009	$r = 0.47$ $p < 0.0001$
IL-17	16.5	2.5	5.2	50	0	365	$r = 0.15$ $p = 0.15$
IL-4	15.4	0.8	4.0	34	0	228	$r = 0.14$ $p = 0.19$
IL-5	34.5	1.7	6.7	175	0	1362	$r = 0.13$ $p = 0.23$
IL-10	2.1	0.25	2.5	3.5	0	18.9	$r = 0.24$ $p = 0.02$
IL-13	134	14	8.5	651	0	5983	$r = 0.14$ $p = 0.20$
IL-12	0.3	0	4.3	1.5	0	8.0	$r = -0.03$ $p = 0.80$
IFN- γ	0.03	0	8.1	0.2	0	1.8	$r = 0.14$ $p = 0.20$