

Staphylococcal Enterotoxin-B-Mediated Stimulation of Interleukin-13 Production as a Potential Aetiologic Factor in Eczema in Infants

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Key Words

Interleukin-13 · Atopic dermatitis · Staphylococcal enterotoxin B

Abstract

Background: *Staphylococcus aureus* colonization has been found in 80–100% of lesional skin from patients with atopic eczema dermatitis syndrome (AEDS) and is thought to have a role in the pathogenesis of the disease. Furthermore, up to 65% of *S. aureus* from lesional skin has been shown to produce toxigenic superantigens.

Methods: Using a cohort of 11 children under 2 years of age diagnosed with AEDS, we isolated peripheral blood mononuclear cells, cultured them with staphylococcal enterotoxin B (SEB) and phytohaemagglutinin, and assessed the cytokine response profiles. Plasma was also collected for immunoglobulin E analysis. In addition, skin and nasal swabs were taken and cultured to determine the presence of SEB-producing *S. aureus* by polymerase chain reaction (PCR) and reverse passive latex agglutination. **Results:** We found a significant increase in the production of the SEB-induced cytokines interleukin (IL)-5 and IL-13 in the patient group when compared with non-atopic, healthy controls. For IL-13, there was almost no overlap in the levels between the groups. However, there was no correlation between SEB-induced IL-13 and disease severity. This difference was not seen when

heat-inactivated *S. aureus* was used to stimulate the cells. **Conclusions:** IL-13 is an important factor in AEDS development in early childhood, and prophylactic anti-staphylococcal treatment may provide protection from AEDS in atopic individuals.

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Introduction

The increasing incidence of atopic disease in developed countries over the last 20 years is now well documented, with the prevalence of atopic eczema dermatitis syndrome (AEDS) in Western countries becoming a major health burden. Figures show that as many as 31% of young children in Australia are affected [1], and a predicted 50% of patients will manifest symptoms before 1 year of age [2]. AEDS is a chronic inflammatory skin condition encompassing a range of clinical phenotypes [3] including the classic form of the disease which is characterized by Th2-biased expression of immunity to allergens involving production of cytokines such as interleukin (IL)-4, IL-5 and IL-13. These cytokines are associated with elevated levels of immunoglobulin E (IgE), increased lesional eosinophilia and activation of mast cells. However, in common with other atopic diseases, many factors are thought to influence the development and severity of the disease including genetic, environmental and immu-

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nological factors [4] together with the competency of the skin as a barrier. The contributions of these factors to AEDS pathogenesis remain poorly understood.

At birth, Th1 responses are attenuated, and it has been suggested that a defect in the postnatal maturation of Th1 function(s) may be a contributing factor in the generation of Th2 memory responses in atopic children [5]. In AEDS, there is evidence that bacteria which colonize the skin may also have a pathogenic role. *Staphylococcus aureus* has been isolated from 80–100% of lesional skin from AEDS patients [6, 7]. Moreover, several studies suggest that a high proportion of the *S. aureus* isolates from AEDS subjects produce toxigenic superantigens (SAg) [8, 9], but this finding is not universal [7]. Superantigens non-specifically stimulate all CD4+ T cells bearing selected T cell receptor (TCR) V β -chains by cross-linking the TCR to an MHC class II molecule. Consequently, they are able to stimulate up to 25% of CD4+ T cells. Stimulation with SAg is characterized by high levels of Th1 cytokine gene expression in normal healthy individuals [10]. However, we have recently shown that peripheral blood mononuclear cells (PBMC) from adults with active AEDS produce high levels of IL-5 in response to the SAg staphylococcal enterotoxin B (SEB) in vitro when compared with non-atopic controls [11]. Although staphylococcal colonization of AEDS patients is higher than in healthy individuals, it is unclear whether all strains of *S. aureus* found produce SAg. The data addressing this issue are conflicting with some reporting an increase in toxigenic strains in childhood and adult AEDS [8, 9] and some finding no difference [12].

The situation in very young age groups is even less clear. Accordingly, the aim of this study was to determine whether any relationship exists between immune responses to staphylococcal superantigens, disease severity and carriage rates of toxigenic *S. aureus* in infants.

Materials and Methods

Study Subjects

Eleven infants with AEDS were included in the study based on the diagnostic criteria of Hanifin and Rajka [13] and disease severity rating of SCORAD >20 [14]. Seven non-atopic, healthy children were recruited as controls. All subjects were between 5 and 25 months of age at the time of sample collection, and all had approximately 10 ml of peripheral blood taken with 2 or 3 skin swabs and a nasal swab. Informed parental/guardian consent was obtained prior to all sample collection. The study design was approved by the human ethics committee of Princess Margaret Hospital, Perth, Western Australia.

Preparation of PBMC

Blood samples were collected into an equal volume of RPMI-1640 (Invitrogen, Australia) containing 20 IU/ml preservative-free heparin (David Bull Laboratories, Australia), and plasma was collected for subsequent IgE analysis following centrifugation and stored at -80°C . The mononuclear cells were isolated by density centrifugation by layering onto LymphoprepTM (Axis-Shield PoC AS, Oslo, Norway), then cryopreserved in 7.5% dimethyl sulphoxide.

Cell Culture

The cells were thawed rapidly and resuspended at 1×10^6 cells/ml in AIM-V serum-free medium (Gibco BRL[®], Life Technologies, Australia), supplemented with 2-mercaptoethanol (Sigma-Aldrich, Australia) at a final concentration of 4×10^{-5} M.

The cells were cultured in either media alone or with SEB (Sigma-Aldrich) at 200 ng/ml, phytohaemagglutinin (PHA; Murex Biotech Ltd., Dartford, UK) at 1 $\mu\text{g}/\text{ml}$ or heat-inactivated *S. aureus* (hiS) at a final concentration of 3.5×10^5 CFU/ml. The cultures were incubated for 48 h at 37°C in a humidified 5% CO_2 environment (Water-Jacketed incubator 3250, Forma Scientific, Marietta, Ohio, USA). Culture supernatants were then collected and stored at -20°C for batch cytokine protein analysis.

Cytokine Analysis

Time-resolved fluorometry (TRF) was used to measure IL-10, IL-13 and IFN γ , and ELISA was used for IL-5. The antibody pairs used have been published elsewhere [15]. Cytokine standard curves were generated using serial dilutions of recombinant human IL-10, IL-13, IFN γ and IL-5 (PharMingen, Pittsburgh, Pa., USA). TRF plates were read on the Wallac² Vicor 1420 multilabel counter (Perkin-Elmer Life Sciences, USA). The ELISA plates were read on a Biotek plate reader (EL-311 Microplate Autoreader, Biotek, Muskegon, Mich., USA).

CD4/CD8 Depletions

Patient PBMC were thawed into cold RPMI with the addition of deoxyribonuclease 1 (Sigma-Aldrich) at a final concentration of 10 $\mu\text{g}/\text{ml}$. The cells were divided; in one half, the CD4+ fraction was depleted, and the other half had the CD8+ fraction removed using the appropriate concentrations of anti-CD4 and anti-CD8 Dynal beads (Dynal Biotech Pty Ltd., Carlton South, Australia), according to the manufacturer's instructions. The resulting populations were stained for the presence of the depleted fractions and found to be >97% pure. The cells were resuspended at 1×10^6 cells/ml in AIM-V and cultured with SEB as described above. By staining the PBMC prior to and after depletion, it was possible to determine the proportion of CD4+ and CD8+ cells at each step and then calculate the cytokine responses on the basis of the numbers of each cell type present in the cultures.

IgE Analysis

Total IgE levels were determined by a sandwich ELISA technique (ImmunoCap[®], Pharmacia Upjohn, Kalamazoo, Mich., USA). Allergen-specific IgE levels were determined by fluorescent enzyme immunoassay (ImmunoCap).

Isolation, Quantitation and Identification of *S. aureus*

The AEDS patients had lesional, non-lesional and nasal swabs taken, whereas the controls had the non-lesional and nasal swabs only, using sterile cotton swabs (Transtube[®], Medical Wire and

Table 1. Characterization of AEDS study subjects and controls

Study	Gender	Age at sampling months	SCORAD	Elevated specific or total IgE	<i>S. aureus</i> carriage		
					lesional	non-lesional	nasal
001	F	10.23	37	+	–	–	+
002	M	10.69	27	+	+	–	+
003	M	17.43	42	+	–	–	–
004	F	5.16	32	+	–	–	–
005	M	17.69	37	+	–	–	+
006	F	23.99	59	+	+	+	+
007	F	12.36	26	+	–	–	–
008	F	12.36	21	–	–	–	–
011	F	11.82	25.5	+	–	–	–
012	F	25.92	60	+	+	+	–
013	M	19.50	37	+	–	–	+
c001	F	8.07	n/a	–	–	–	–
c002	M	12.69	n/a	–	–	–	–
c003	F	13.26	n/a	–	–	–	–
c004	F	12.79	n/a	–	–	–	–
c005	F	13.10	n/a	–	–	–	+
c006	F	12.17	n/a	–	–	–	–
c007	M	12.92	n/a	–	–	–	–

The CAP RAST allergen panel comprises mixed foods (FX2), mixed grasses (GX2), *Dermatophagoides pteronyssinus*, egg white, cow's milk, wheat flour, peanut, soya bean and SEB. Samples above 0.35 kU/l were designated as elevated. For total IgE, the cut-off for the positive reference range is determined for each assay, and results above the cut-off for respective assays were designated as elevated. Only 1 patient (002) was RAST positive for SEB.

Equipment Company, UK) pre-moistened by immersion in 1 ml sterile saline solution. A template was utilized to delineate a 25-cm² sampling area. All swabs were stored at 4°C overnight until processing the next day. The swabs were placed in 2-ml aliquots of tryptic soy broth and vortexed for 1 min. To quantify the number of *S. aureus* present in the swab, 50 µl of swab fluid was plated out onto non-selective blood agar plates and selective MacConkey sorbitol agar plates and incubated for 24 h at 37°C. A positive identification of *S. aureus* was confirmed with 3 tests: (1) Staphatech® (Oxoid, Basingstoke, UK) latex agglutination assay, (2) DNase assay (Oxoid) and (3) coagulase activity assay (Oxoid), all performed according to the manufacturer's recommendations.

Detection of Toxigenic *S. aureus*

The *S. aureus* isolates from the swabs were tested by staphylococcal enterotoxin-reverse passive latex agglutination (Oxoid) using the method recommended by the manufacturer for the presence of staphylococcal enterotoxins (SE): A (SEA), SEB, SEC, SED and toxic shock syndrome toxin-1. The presence of SEB was also determined by conventional PCR using the SYBR Green® Mastermix system (Applied Biosystems, Scoresby, Australia). Bacterial genomic DNA was prepared using a Qiagen DNA Mini Kit (Qiagen, Australia). The following primers, used at 200 nM, were obtained from Geneworks (Australia) and designed using Primer Express™ software (Version 1.0, Applied Biosystems); forward 5'-TGA ATT TAA CAA CTC GCC TTA TGA A-3' and reverse 5'-TGT ACA ATG ACA ATA AAA TGG TTG AT-3'. An SEB-positive strain of *S. aureus* used as a

positive control was a kind gift from Frances O'Brien, Medical Research Facility, Royal Perth Hospital, NCTC 10654. The PCR was carried out in a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems). The presence of the SEB gene was confirmed by sequencing (Supamac®, Royal Prince Alfred Hospital, Sydney, Australia).

Statistical Analysis

For non-normally distributed data, as defined by a significant p value returned for the Kolmonorov-Smirnov normality test, Mann-Whitney U tests were performed. For normally distributed data, the parametric equivalents were used. The software package Statview (Version 5.0.1, SAS Institute Inc., Cary, N.C., USA) was utilized, and all graphs were generated using Prism (Version 3.0a, GraphPad Software Inc., San Diego, Calif., USA).

Results

Details of the study subjects are shown in table 1. The mean age of the patient group was 15.4 months (n = 11) and for the controls 12.14 months (n = 7). The ratio of males to females in the patient group was 1:1.75, with 1:2.5 in the controls.

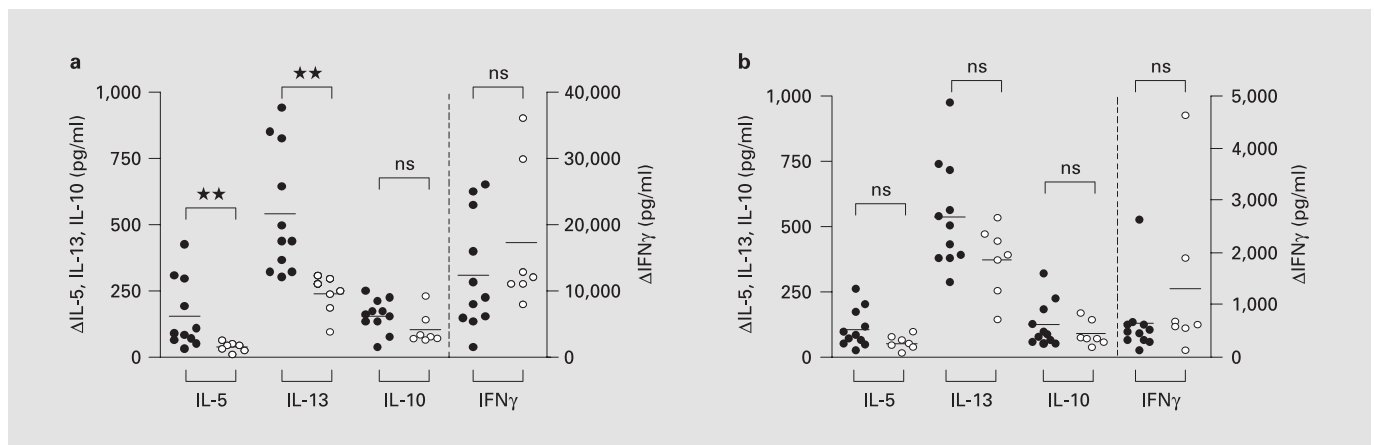


Fig. 1. Cytokine produced in response to SEB and PHA. PBMC from patients (●) or controls (○) were cultured for 48 h alone or with (a) SEB or (b) PHA. The culture supernatants were harvested and analysed for the presence of IL-5, IL-13 and IL-10 on the left axis, and IFN- γ on the right axis. Values were corrected for background levels. * $p < 0.05$; ** $p < 0.005$. As the data were normally distributed, the means are shown.

Table 1 shows the numbers of children who were colonized with *S. aureus* and swabbed at 3 different sites, non-lesional, lesional and nasal. Fifty-five percent of the patient group were colonized with *S. aureus* at 1 or more sites. Carriage of *S. aureus* on lesional skin was seen in 27% of patients, and 45% carried *S. aureus* in the nasal passages. SEB-expressing *S. aureus* was detected in 18% of patients.

Total and allergen-specific IgE analysis from the serum of the study subjects was carried out to a panel of food and environmental allergens. Six of the 11 patients were sensitized to 1 or more of the allergens tested compared with the controls where no sensitization was detected (data not shown). Of those sensitized, food allergens were most commonly involved. Six of the patients and 2 controls had total IgE levels in serum at $\geq$ double the reference cut-off.

Analysis of Cytokine Release from PBMC in Response to SEB and PHA

The polyclonal cytokine responses of the patient and control groups are illustrated in figure 1. The most striking feature of these results is the level of SEB-induced IL-13 in the patient group compared with the controls, with minimal overlap in the respective response ranges of the 2 groups (mean IL-13 = 540 pg/ml for the patients and 237 pg/ml for the controls; $p = 0.005$). The expression of IL-5 in the culture supernatants was also significantly increased in response to SEB in the patient group (mean IL-5 for the patients = 147.5 pg/ml, with 39.14 pg/ml for

the controls; $p = 0.022$). In contrast, there was no significant difference between the groups in the IL-10 and IFN- γ responses to SEB. Moreover, the PHA-induced cytokines did not differ between the 2 groups. Also worthy of note is the significantly increased IFN- γ production in response to SEB compared with PHA in both the patients and the controls (patient group mean SEB IFN- γ = 12,508 pg/ml, mean PHA IFN- γ = 636 pg/ml; $p = 0.0008$); in contrast, SEB induced significantly lower levels of IL-13 than PHA, but only in the controls (SEB IL-13 = 237 pg/ml, PHA IL-13 = 371 pg/ml; $p = 0.0031$).

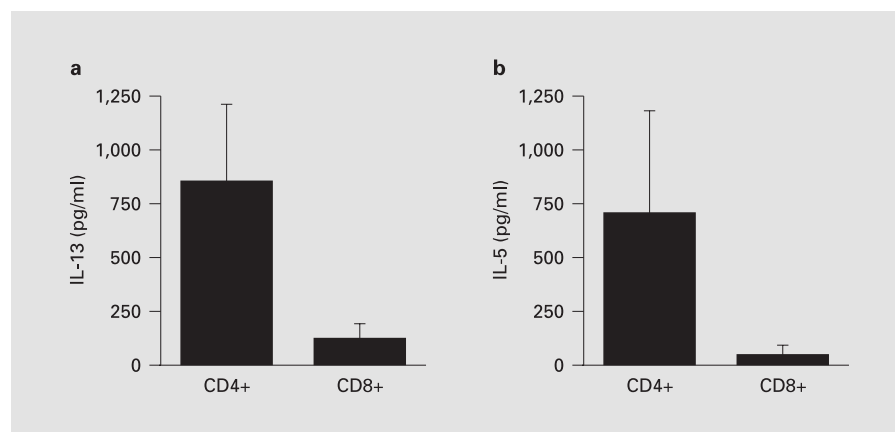
Identification of the T Cell Population Producing the Increased IL-13 and IL-5 in Response to SEB

In an attempt to identify the cells which were responding to SEB with increased IL-13 and IL-5 production, PBMC were depleted of CD4+ cells or CD8+ cells and subsequently cultured in the presence of SEB. After 48 h, the supernatants were collected and assayed for IL-13 and IL-5. The results are shown in figure 2. These experiments clearly identify CD4+ T cells as the principal source of SEB-induced cytokines.

Cytokine Responses to hiS

We additionally cultured patient and control PBMC with a preparation of hiS for 48 h and then performed TRF or ELISA on the culture supernatants to assess the levels of cytokines produced. It is clear from the data presented in figure 3 that there were no significant differences in the level of cytokines produced between the 2

Fig. 2. IL-13 and IL-5 production by CD8- or CD4-depleted PBMC in response to SEB. PBMC from patients were depleted of either CD4+ cells or CD8+ cells, made up to 1×10^6 cells/ml and cultured with SEB for 48 h. The culture supernatants were collected and analysed for the presence of IL-13 (a) and IL-5 (b). The figures were adjusted to account for the different numbers of CD4+ or CD8+ cells present in the cultures. This is the result from 3 patients.



groups. This finding further emphasizes the potential importance of SEB as opposed to other *S. aureus*-derived antigens in driving the expression of the AEDS phenotype.

Discussion

It is clear that Th2-type cytokines are of fundamental importance in atopic disease, with IL-5 involved in eosinophil recruitment and maturation, and IL-4 and IL-13 functioning as key regulators of IgE production [16, 17]. Of direct relevance to this study, Ohshima et al. [18] have presented evidence that increased IL-13 production by CD4+ mononuclear cells in cord blood is associated with increased risk for subsequent development of atopic disease. CD8+ T cells in cord blood have also been identified as producers of high levels of IL-13 detected following in vitro stimulation with anti-CD3, anti-CD28 and IL-2 [19]. This suggests an intrinsic predisposition of neonatal CD8+ T cells to secrete high levels of IL-13, consistent with the observed Th2 bias of the neonatal immune system. In studies using RT-PCR, IL-13 has been shown to be increased in PBMC of adult AEDS patients compared with healthy controls [20], and in investigations utilizing intracellular cytokine staining and flow cytometry, an increase in the frequency of both CD4+ and CD8+ T cells expressing increased levels of IL-13 in adult patients was demonstrated [21]. Moreover, a recent study in adults has demonstrated increased IL-13 in PBMC from adult AEDS patients in response to anti-CD3/CD28 stimulation [22].

Here, we reveal for the first time a link between SEB and increased IL-13 in AEDS in infants. When the PBMC

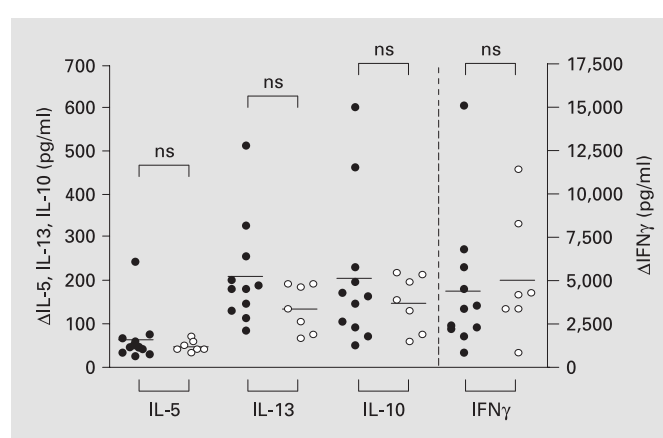


Fig. 3. Cytokine produced in response to hiS. PBMC from patients (●) or controls (○) were cultured for 48 h alone or with hiS. The culture supernatants were harvested and analysed for the presence of IL-5, IL-13 and IL-10 on the left axis, and IFNγ on the right axis. Values were corrected for background levels. As the data were normally distributed, the means are shown.

from the study group were cultured with SEB, the IL-13 response observed was significantly higher in the patient group compared with the controls; moreover, the 2 groups showed very little overlap in levels of expression. We found no correlation between IL-13 production and disease severity, which suggests that IL-13 is necessary for disease, but other factors, as yet undefined, may determine severity. Smart and Kemp [23] found no significant difference in SEB-induced IL-13 expression between atopics and non-atopics in the same age bracket, but the subjects were not exclusively AEDS patients, which suggests that the increase we are seeing may be particular to AEDS. Prescott et al. [24] also showed increased IL-13 in

atopics in response to allergen-specific stimulation at 2 years of age, indicating that there is an increased propensity for IL-13 production in early childhood, specifically related to atopic disease development. In our study, house dust mite responses were measured, but they were below the level of detection (data not shown).

SEB-induced levels of IFN γ from PBMC of individuals from both groups in this study were significantly increased when compared with PHA-induced levels. SAg are known to activate T cells displaying specific variable regions on the TCR β -chain, and in AEDS, preferential use of V β 8 has been identified in lesional T cells [25]. SAg stimulation can activate up to 25% of the T cell compartment, while PHA stimulates all T cells non-specifically, theoretically up to 100% of T cells. The findings presented in figure 1 suggest that the SEB-induced cytokine responses can therefore be more than 4 times the magnitude of the PHA-induced responses. The significant differences between SEB and PHA-induced IFN γ in the control population are not unexpected, as microbial antigens and superantigens specifically induce a Th1 response in normal healthy individuals [10]. The control subjects showed significantly lower levels of IL-13 in response to SEB than to PHA, reflecting the Th1 bias of SAg stimulation, in contrast to the patients where there was no difference in the IL-13 response to either stimuli, but production of this cytokine was increased when compared with the controls, confirming the inherent differences in the T cell responses between the patients and controls.

The IL-5 levels detected in response to SEB in culture supernatants were also significantly increased in the infant patient group, which is consistent with what we have shown previously in adults [11]. Regarding the IFN γ response of the 2 groups, no significant differences were found; however, the mean IFN γ levels were decreased in the patient group. This result was also seen in the previously mentioned study [23], where there was a significant decrease in IFN γ response to SEB in atopics, in general below the age of 2 years, compared with non-atopics. Other studies [26, 27] have demonstrated a similar decrease in atopic and non-atopic children of unrestricted age.

We did not find any significant correlations between disease severity and immune outcomes measured within the patient group. Only 2 of the 11 patients were positive for SEB-producing *S. aureus*, which was not enough for meaningful comparisons; however, the overall *S. aureus* colonization rates of the patients were markedly higher than those of the controls (55% vs. 14%). This finding supports the theory that atopic skin is fundamentally able to support higher colonization levels of *S. aureus*, which

may be related to a reduction in anti-staphylococcal lipids within the skin [28] or reduced levels of innate response proteins, such as human β -defensins (HBD) which are secreted by human keratinocytes [29]. Nomura et al. [30] have shown that the level of HBD-3, a recently identified anti-microbial peptide, is lower in AEDS skin than in skin of psoriasis patients. Furthermore, they demonstrated a negative correlation between HBD-3 and IL-13 expression in AEDS. Numerous studies have shown a similar trend with colonization rates in AEDS [7, 9, 31, 32]; however, most of these studies describe colonization in $\geq 80\%$ of patients and $\leq 30\%$ of healthy controls. The most likely reason for this discrepancy with our data is the different age groups and population size studied.

Mean total IgE levels in the patient group were considerably higher than those of the controls; however, allergen-specific IgE levels were extremely variable. Notably, approximately half of the AEDS group were RAST negative to the large allergen panel, which suggests that the observed relationship between positive SCORAD and Th2-biased hyperresponsiveness to SEB may be an intrinsic feature of the immune response phenotype of these infants, and not secondary to underlying atopy. This suggestion is consistent with our recent findings linking hyperresponsiveness of cord blood mononuclear cells to SEB to subsequent development of atopic dermatitis (AD) [15], and also with the report linking hyperproduction of IL-13 by cord blood cells with AD risk [18].

The precise mechanism(s) underlying the relationship between responsiveness to SEB and skin disease in infants remains to be defined. It is important to note that our studies were by necessity restricted to T cell responses in peripheral blood, which are used as a model to show how similar interactions may occur in skin, but the formal proof that these interactions actually occur in vivo is currently lacking. Despite this caveat, it is nevertheless reasonable to hypothesize that superantigens produced by *S. aureus* at the skin may directly activate T cells which then release pro-inflammatory cytokines locally and/or traffic to draining lymph nodes where they may then create a microenvironment which will enhance the Th2 response to environmental allergens. Epicutaneous allergen exposure together with the bystander effect of SAg stimulation of T cells may then promote the pathogenesis of AEDS. Alternatively, direct stimulation of skin-dwelling T cells by SEB may induce local secretion of pro-inflammatory cytokines and hence inflammation, independent of atopy. We suggest on the basis of the data presented above that these scenarios are sufficiently plausible to merit more detailed investigation of the role of SEB in skin inflamma-

tion in the early postnatal period. Of particular interest is the possibility that prophylactic treatment based on the control of dermal staphylococcal colonization in early life may provide protection from AEDS-like disease in both atopic and non-atopic infants.

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