

Age-Related Differences in Sensitivity of Peripheral Blood Monocytes to Lipopolysaccharide and *Staphylococcus Aureus* Toxin B in Atopic Dermatitis

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As shown by atopy patch tests, atopic dermatitis (AD) is dominated in its acute phase by the development of a specific T_H2 response after exposure of the skin to common environmental antigens. Relying on our previous data showing that *Staphylococcus aureus* enterotoxin B (SEB) induced the activation of monocyte-derived dendritic cells (DCs) through Toll-like receptor (TLR)2 and that SEB-pulsed DCs commit allogenic naive T cells into T_H2, we assessed monocytes sensitivity to SEB and lipopolysaccharide (LPS) in a group of children and adult patients with AD. Monocytes from AD patients (15 adults with mostly severe disease and 15 children with mild to moderate disease) exhibited an activated and tolerant state as supported by (i) secretion of large amounts of IL-6, IL-10, and tumor necrosis factor- α even in the absence of stimulation; (ii) their inability to modulate neither HLA-DR and CD54 nor TLR2 and TLR4 expression after *in vitro* challenge with SEB; (iii) inhibition of IL-12p70 secretion in response to LPS. Interestingly, monocytes from some of the children studied responded to *in vitro* challenge with LPS, suggesting new hypotheses to explain disease regression. Our data support the notion that monitoring sensitivity of monocytes to bacterial toxins could prove useful to assess disease progression and prognosis in AD.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by typically distributed pruritic eczematous skin lesions associated with an hyper-reactivity to environmental triggers (Darsow *et al.*, 2005). The pathophysiology of AD results from a complex interaction of genetics, environmental, and immunological factors, and current research focuses on the interactions between an intrinsically abnormal cutaneous permeability barrier (Taieb, 1999) and the innate immune system (McGirt and Beck, 2006). Especially, the initial maturation steps of the skin barrier in the first months of life are viewed as pivotal to induce a skin-borne skewing of the immune system toward T_H2 responses (Taieb *et al.*,

2006). Besides an increased proneness to react to irritants and to enhance the penetration of allergens, the overall impairment of cutaneous barrier function noted in AD leads to an increased susceptibility to herpes simplex virus and is associated with a colonization by *Staphylococcus aureus*, detected in more than 90% of patients in non-eczematous skin, both events being associated with a decreased production of antimicrobial peptides (Leung *et al.*, 1993; Ong *et al.*, 2002; Howell *et al.*, 2006). More than half of *S. aureus* strains isolated from AD patients are able to produce exotoxins with superantigenic activity such as staphylococcal enterotoxin A, *S. aureus* enterotoxin B (SEB), or toxic shock syndrome toxin-1 (Hoeger *et al.*, 1992). All of these toxins can mediate nonspecific T-cell activation by their superantigenic activity and act as conventional allergens stimulating the production of specific antibodies (McFadden *et al.*, 1993). In some patients, specific IgE antibodies directed against staphylococcal superantigens have been detected in both sera and skin (Leung *et al.*, 1993) that could lead to the release of histamine from basophils and also induce the activation of antigen-presenting cells bearing specific receptors. Interestingly, it has been reported that serum levels of antibodies directed against staphylococcal toxin correlate with disease severity (Zollner *et al.*, 2000).

Professional antigen-presenting cells such as dendritic cells (DCs) and monocyte-macrophages are activated through recognition of pathogen-derived molecular patterns

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Abbreviations: AD, atopic dermatitis; DC, dendritic cell; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; NK, natural killer; SEB, *Staphylococcus aureus* enterotoxin B; TLR, Toll-like receptor

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by innate receptors such as Toll-like receptors (TLRs). Sensing of pathogens by activated monocytes known to express most of the TLRs (Kadowaki *et al.*, 2001) takes part in the development of an adaptive immunity and also permits to control dissemination of infection by inducing local inflammation. Besides recognition of the known bacterial pathogen-derived molecular patterns by TLR, we speculated that cells of the monocyte-macrophage lineage as well as DCs could sense toxins such as those secreted by *S. aureus* and then contribute to an early control of infection. In a previous work, we demonstrated that SEB could induce the maturation of DCs through activation of TLR2 leading to priming of naive allogenic T cells into T_H2 (Mandron *et al.*, 2006), a finding which may pertain to the sequence of events noted in the skin in early AD natural history, since staphylococci found at the skin surface could induce, through toxin production, a signal affecting T-cell commitment.

Our hypothesis, in this study, was that the maturation of SEB-driven innate immune responses could reflect eventually the course and severity of AD. We first show that TLRs can participate to the activation of normal monocytes by SEB, as already shown for monocyte-derived DCs (Mandron *et al.*, 2006). We further provide evidence that, contrary to non-atopic controls, most AD adult patients and a majority of children show an hyperactivated status of their purified monocytes that release spontaneously large amounts of inflammatory cytokines (IL-6, IL-10, and tumor necrosis factor (TNF- α)) but fail to respond to an additional stimulation with SEB. However, we could detect that hyporesponsiveness of AD monocytes to lipopolysaccharide (LPS), as revealed by the absence of bioactive IL-12 (IL-12p70) secretion, was sparing some AD children, and this finding may reflect an essential step in disease progression.

RESULTS

SEB can activate normal human monocytes

To analyze the effect of SEB on human monocytes, peripheral blood monocytes were isolated by magnetic cell sorting using CD14⁺-positive selection and incubated with increasing concentrations of SEB or LPS (1–1,000 ng ml⁻¹) for 24 hours. Cells were then analyzed for surface expression of HLA-DR by flow cytometry (Figure 1a). Figure 1b shows that HLA-DR expression increased with respect to SEB concentration and that a twofold increase of mean fluorescence intensity (MFI) was obtained with as less as 1 ng ml⁻¹ of toxin. Similar results were obtained when using highly purified SEB (data not shown). In contrast, LPS induced a quite low increase of HLA-DR expression. SEB and LPS were used at 10 ng ml⁻¹ final concentration for subsequent investigations. We then analyzed the expression of various markers known to reflect activation of monocytes such as CD54, CD80, and CD86. After treatment with SEB or LPS, a strong upregulation of CD54 and CD80 expression was observed under stimulation with either SEB or LPS (Figure 2) contrasting with a slight inhibition of CD86 expression. These data demonstrated that like LPS, SEB was able to induce activation of human monocytes.

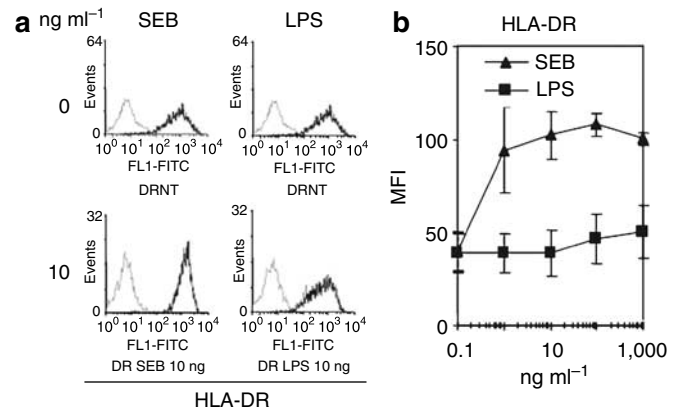


Figure 1. Activation of CD14⁺ monocytes by SEB. (a) Monocytes isolated from non-atopic subjects were treated with increasing concentration of SEB or LPS (0–1,000 ng ml⁻¹). After 24 hours of incubation, HLA-DR expression was analyzed by flow cytometry, and histograms of MFI are shown. (b) Graphic representation of dose-response to stimulations with SEB or LPS. Averages from all the patients tested with standard deviations are shown.

SEB-mediated activation of normal monocytes induces modulation of TLR expression

It is well known that binding of LPS to TLR4 induces activation of inflammatory cells of the monocyte-macrophage lineage, including DCs. We already demonstrated that normal monocyte-derived DCs were able to secrete TNF- α in response to interaction of SEB with TLR2 (Mandron *et al.*, 2006). Moreover, we showed that SEB induced activation of NF- κ B in stably transfected TLR2-HEK293 cells, which do not express MHC (major histocompatibility complex) class II, confirming the ability of SEB to trigger TLR2 signaling.

To investigate whether TLRs could participate in the activation of normal monocytes by SEB, we analyzed TLR2 and TLR4 expression on cells after stimulation with increasing doses of toxin. Cytometric analysis of TLR2 and TLR4 protein expression at the surface of untreated monocytes revealed a constitutive expression of both receptors. Treatment with SEB induced a significant upregulation of TLR2 and TLR4 expression even at low SEB concentration (10 ng ml⁻¹) (Figure 3a). To clarify the role of TLR2 and TLR4 in SEB-induced activation of monocytes, cells were pretreated with neutralizing antibodies against TLR2 or TLR4 before SEB or LPS stimulation and analyzed for TNF- α secretion. Figure 3b shows that after 24 hours of stimulation, SEB-treated monocytes were able to produce TNF- α and at a higher level than those treated with LPS. When monocytes were pretreated with either anti-TLR2 or anti-TLR4 neutralizing antibodies before incubation with SEB, a significant decrease in TNF- α secretion was observed. As we expected, TNF- α secretion following stimulation with LPS was abolished only in the presence of TLR4 neutralizing antibody. When both anti-TLR antibodies were used, as well as when anti-MHC class II antibodies were added to the mixture, no extra neutralization was observed. This may be explained for instance by (i) saturation of MyD88 or TRAF6 recruitment when any of the TLR2 or TLR4 is triggered and (ii) the absence

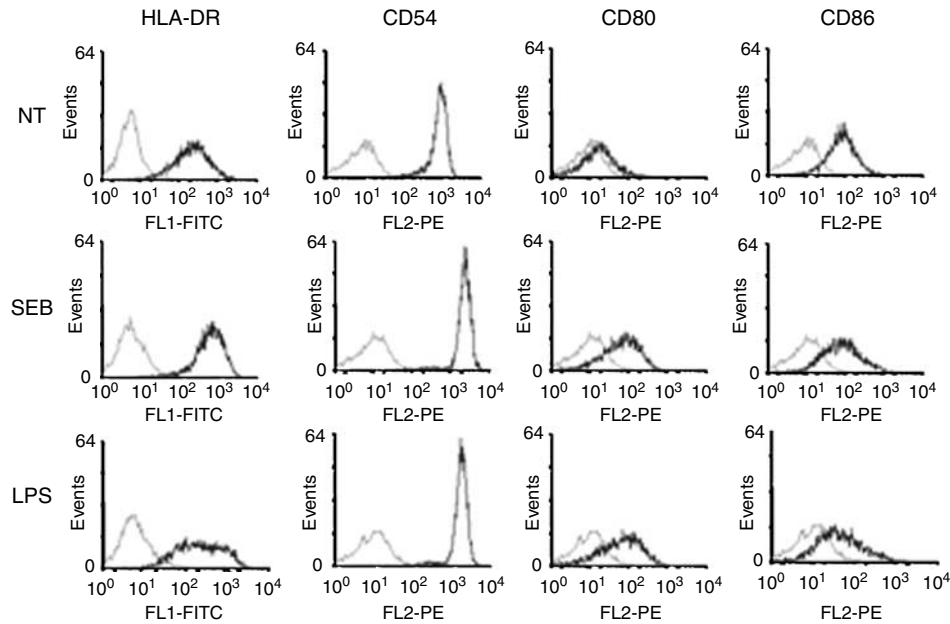


Figure 2. Phenotype of SEB-activated monocytes. Monocytes were cultured in the presence of 10 ng ml^{-1} of SEB or LPS or left untreated (NT) for 24 hours. HLA-DR, CD54, CD80, and CD86 surface expression was analyzed by flow cytometry as described in the Materials and Methods section.

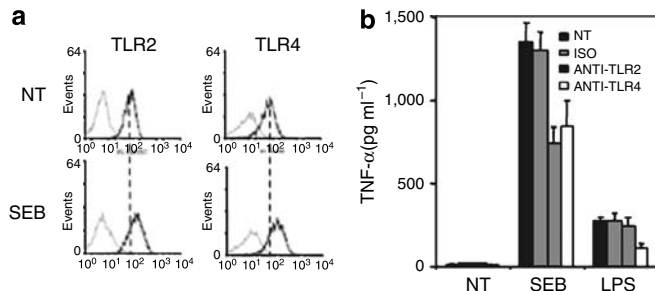


Figure 3. Modulation of TLR2 and TLR4 expression on activated monocytes. (a) Monocytes were either untreated (NT) or incubated for 24 hours with 10 ng ml^{-1} of SEB or LPS. Surface expression of TLR2 and TLR4 was analyzed by flow cytometry, and histograms of MFI are shown. (b) Monocytes were preincubated with neutralizing antibodies to TLR2 or TLR4 or isotypic control. Cells were treated for 24 hours with 10 ng ml^{-1} of SEB or LPS, and TNF- α secretion was analyzed by ELISA on culture supernatants.

of TNF- α secretion through MHC class II in conditions used in our experiments (low SEB concentration...). These results suggested that interaction of SEB with both TLR2 and TLR4 could lead to monocyte activation as revealed by both upregulation of activation markers and secretion of TNF- α .

Monocytes from AD patients are hyperactivated and desensitized to SEB stimulation

In non-atopic individuals, colonization of the epidermis by *S. aureus* is controlled by cutaneous immunity, and we can speculate that in atopic patients with high colonization the early recognition of pathogens by sentinel cells such as monocytes and DCs could be impaired. Assuming a role for

monocytes or monocyte-derived cells in sensing *S. aureus*, we asked whether variations in sensitivity of atopic patients to *S. aureus* toxins could reflect those dysfunctions. Sensitivity to stimulation with SEB or LPS (10 ng ml^{-1}) of monocytes isolated from 30 AD patients was compared with that of monocytes from 15 non-atopic controls, as assessed by flow cytometry analysis of HLA-DR expression.

Figure 4 shows a strongly significant ($P < 0.001$) upregulation of HLA-DR on monocytes of non-atopic individuals after treatment with SEB, contrasting with a barely detectable modulation on those from AD patients ($P > 0.05$). Furthermore, we observed a stronger constitutive expression of HLA-DR on monocytes from atopic children than on those from non-atopic adult. This may reflect an activated stage of monocytes from AD patients, which is likely due to their inflammatory status. To assess whether upregulation of HLA-DR on NAD (non-atopic dermatitis) monocytes could depend on the presence of IFN- γ secreted by contaminating activated T cells in the monocyte preparation, cocultures of monocytes (Mo) with autologous T cells (T) were performed at T to Mo ratios ranging from 0.01 to 1. Cocultures were treated with SEB (10 ng ml^{-1}) for 24 hours and IFN- γ was quantified by ELISA. At T to Mo ratios from 0 to 0.1, the average amounts (five independent experiments) of secreted IFN- γ ranged from 50 to $3,500 \text{ pg ml}^{-1}$, respectively, and reached $20,000 \text{ pg ml}^{-1}$ at 1:1 ratio (data not shown). Then, equivalent amounts of recombinant IFN- γ were added to purified monocytes in the absence of SEB, and HLA-DR expression was analyzed by flow cytometry. In these conditions, no upregulation of HLA-DR expression was observed except at the highest amount of IFN- γ used but in a low percentage of cells, demonstrating that upregulation of HLA-DR by SEB was

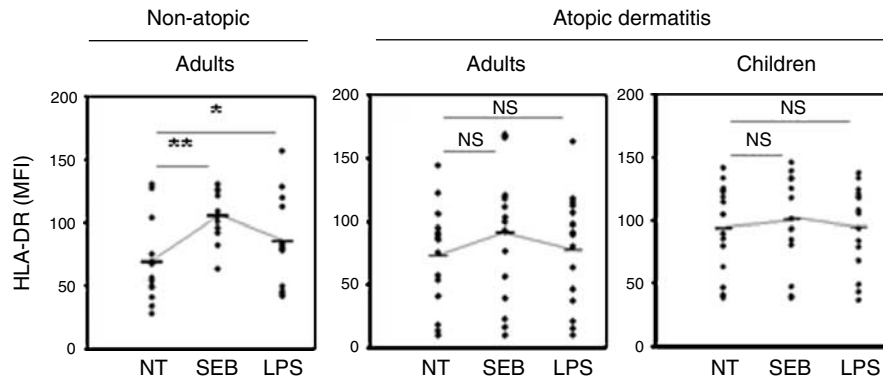


Figure 4. Activation of monocytes from atopic (AD) patients. Monocytes from 30 AD patients (15 adults and 15 children) or 15 non-atopic controls as indicated were cultured for 24 hours in the presence of 10 ng ml^{-1} of SEB or LPS. Surface expression of HLA-DR was analyzed by flow cytometry, and histograms of MFI values are shown after deduction of MFI values corresponding to isotypic controls. Mean values of MFI for each experiment are artificially connected and compared according to paired Student's *t*-test (* $P < 0.005$, ** $P < 0.001$, NS, not significant).

not due to contaminating T cells, which never represented more than 1% of monocyte preparations.

As shown by cytokine production, AD monocytes are hyperactivated and cannot respond to an additional *in vitro* stimulation with SEB

Activation of monocytes leads to the secretion of cytokines that are important for the control of pathogen colonization and the development of appropriate immune responses. We used a cytometry bead array assay to investigate secretion of inflammatory cytokines by monocytes either untreated or incubated with SEB or LPS. Monocytes isolated from non-atopic individuals (NADs, Figure 5) secreted the inflammatory cytokines IL-6 and TNF- α and the regulatory cytokine IL-10 under treatment with SEB but at a lower level than those from atopic patients (ADs), suggesting that AD monocytes have been activated *in vivo* and could not respond to an additional *in vitro* stimulation with SEB.

TLR expression on monocytes is not modulated by SEB in AD patients

Then, we analyzed the expression of TLR2 and TLR4 after stimulation of monocytes with SEB or LPS (10 ng ml^{-1} for 24 hours), by flow cytometry. Figure 6 shows that expression of TLR2 on AD monocytes was unchanged after exposure to SEB in contrast with a strong upregulation in non-atopic subjects ($P < 0.001$). Similarly, TLR4 expression was not enhanced after exposure to SEB.

Age-related differential production of IL-12 by monocytes isolated from AD patients in response to LPS

IL-12p70 secretion is a crucial factor in the establishment of immunity against pathogens because it drives the development of T_H1 responses. In a previous study, we demonstrated that mature DCs were not able to produce IL-12p70 in response to stimulation with SEB and committed naive allogenic T cells into T_H2 (Mandron *et al.*, 2006). Accordingly, we asked whether monocytes from AD patients were able to secrete bioactive IL-12 in response to LPS and SEB. Secretion of IL-12p70 was quantified by ELISA on non-atopic monocytes and compared with that from AD patients. As

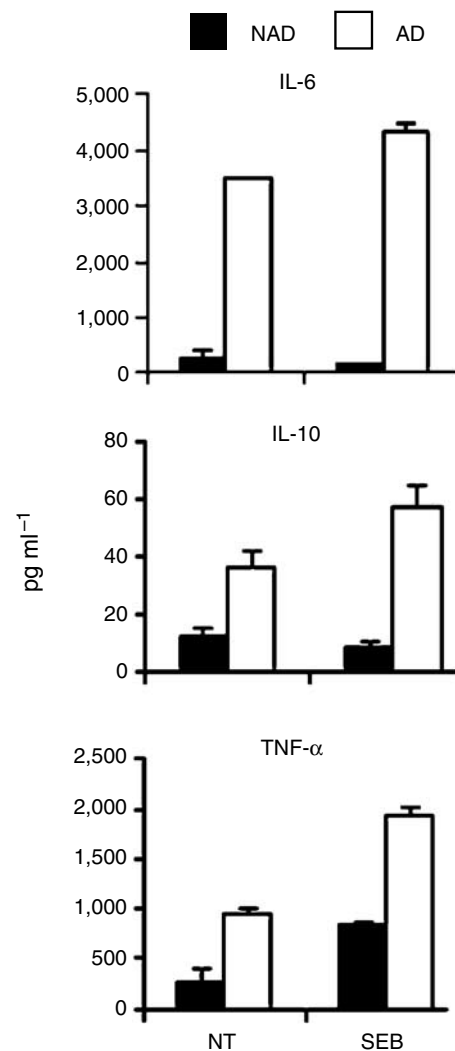


Figure 5. Production of cytokines by atopic monocytes. Monocytes isolated from AD patients and non-atopic controls were treated with 10 ng ml^{-1} of SEB or LPS. Supernatants collected after 24 hours of incubation were analyzed for cytokine production using cytometry bead array assay. Histograms show concentration (pg ml^{-1}) for IL-6, IL-10, and TNF- α cytokines secreted by monocytes from 10 different adult individuals either non-atopic (NAD) or atopic (AD).

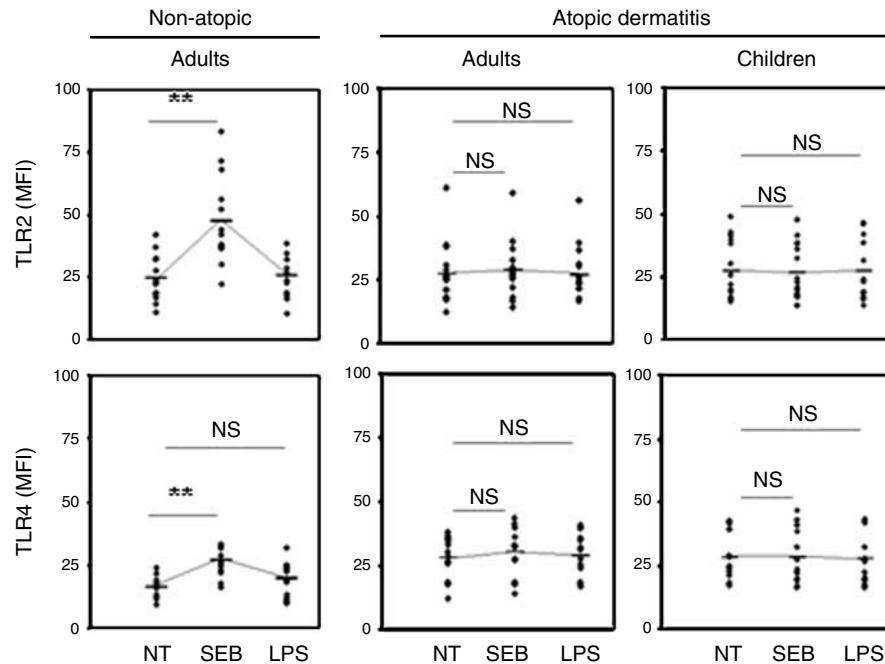


Figure 6. Modulation of TLR2 and TLR4 expression on atopic monocytes. Cultures of monocytes from 30 AD patients or 15 non-atopic controls were either untreated (NT) or cultured in the presence of 10 ng ml^{-1} of SEB or LPS for 24 hours. Surface expression of TLR2 and TLR4 was analyzed by flow cytometry using specific monoclonal antibodies. Histograms of MFI values are shown after deduction of MFI values corresponding to isotypic controls. Mean values of MFI for each experiment are artificially connected and compared according to paired Student's *t*-test (** $P < 0.001$, NS, not significant).

expected, stimulation of monocytes from non-atopic subjects with LPS induced IL-12p70 secretion (Figure 7). On the opposite, no IL-12p70 secretion was observed after stimulation of these cells with SEB confirming our previous results obtained on DCs (Mandron *et al.*, 2006).

We then determined the ability of monocytes from adult patients to produce the bioactive form of IL-12. Neither untreated nor SEB-treated adult AD monocytes produced significant levels of IL-12p70 as observed in non-atopic subjects. Interestingly, under stimulation with LPS, IL-12p70 production was significantly lower in AD monocytes than in non-atopic controls.

We next analyzed the capacity of monocytes from AD children to secrete IL-12p70. Similarly to monocytes isolated from non-atopic subjects or adult atopic patients, untreated as well as SEB-treated monocytes isolated from atopic children did not produce significant amounts of IL-12p70. Nevertheless, contrary to adult patients, some of the children examined in this study (inset in Figure 7) were able to respond to LPS stimulation and produced amounts of IL-12 within the same range as that observed in non-atopic adult individuals.

DISCUSSION

Skin colonization by *S. aureus* is a key factor in the pathogenesis of AD, in spite of the limited therapeutic impact of anti-staphylococcal intervention in established disease. One of the strategies whereby *S. aureus* exacerbates AD is by secreting toxins able to stimulate lymphocytes through their superantigenic activity, thus creating strong inflammatory conditions leading to an increased bacterial colonization of skin.

Besides their known superantigenic effects on T lymphocytes, we assumed that owing to their ability to gain access to epidermal monocytes and DCs, exotoxins could take part in the early steps of the disease in local inflammation of still undamaged skin. Assuming that interactions of toxins with monocytes could result in secretion of cytokines leading to an inappropriately biased response of T lymphocytes, monocytes from non-atopic subjects and AD patients were isolated from peripheral blood using magnetic cell sorting and incubated with SEB or LPS. Then, according to our previous data demonstrating a role for TLR2 in activation of DCs by SEB, we analyzed the capacity of SEB to induce activation of monocyte and to modulate TLR2 and TLR4 expression. In this report, we demonstrate that in non-atopic subjects activation of monocytes with SEB resulted in an upregulation of HLA-DR, CD80, and CD54. Analysis of TLR expression revealed a strong constitutive expression of both TLR2 and TLR4 that was upregulated after exposure to SEB. Secretion of TNF- α was partly but significantly blocked when cells were pretreated with TLR2 and TLR4 neutralizing antibodies, demonstrating a key role for SEB-mediated signaling of TLR in activation of monocytes. Regarding the absence of DCs activation by SEB through TLR4 in our previous study (Mandron *et al.*, 2006), we can assume that it was due to a lower expression of TLR4 and CD14 on monocytes-derived DCs than on monocytes. Monocytes isolated from atopic patients were constitutively activated and resisted to subsequent *in vitro* stimulation as assessed through analysis of both activation markers and cytokines secretion. Accordingly, stimulation of monocytes from AD patients with SEB

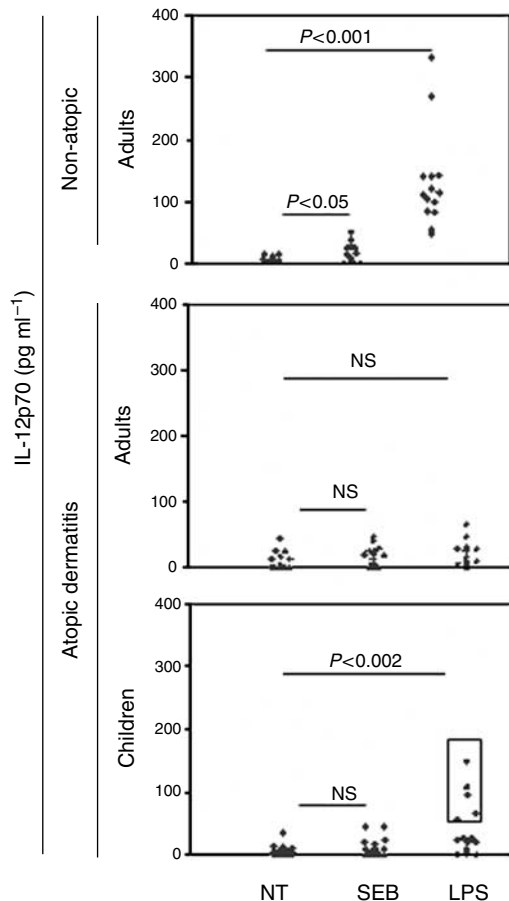


Figure 7. Production of IL-12 by atopic monocytes. Monocytes isolated from 30 AD patients (15 adults and 15 children) and 15 non-atopic controls were either untreated (NT) or treated with 10 ng ml⁻¹ of SEB or LPS for 24 hours. Secretion of IL-12p70 was subsequently analyzed by ELISA on culture supernatants and compared according to paired Student's *t*-test.

did not modulate expression of TLR2 and TLR4 at their surface.

TLR stimulation is crucial for activation of innate immunity and for the control of an appropriate adaptive response devoted to pathogen elimination. This study demonstrating that both TLR2 and TLR4 can be involved in recognition of SEB invites us to consider kinetics and outcomes of TLR activation with respect to progression and severity of AD. This assumption is supported by a previous report demonstrating a decreased expression of many antimicrobial gene products, including human β -defensin-3, TNF- α , and IFN- γ in AD skins, providing an explanation for their increased susceptibility to microorganisms (Nomura *et al.*, 2003), and by studies demonstrating a predictive risk for subsequent AD development in children with specific patterns of responsiveness by cord blood monocytes to SEB (Sharp *et al.*, 2003). Our observation that monocytes from AD patients are desensitized to activation by LPS is in agreement with the reported endotoxin tolerance in human and mice (Nomura *et al.*, 2000; Wysocka *et al.*, 2001) as characterized by IL-12 suppression. Indeed, we can speculate that *in vivo* activation of AD monocytes by SEB could make them refractory to

in vitro restimulation. Endotoxin tolerance in mouse macrophages was shown to partly correlate with downregulation of TLR4 expression (Nomura *et al.*, 2000). Moreover, an association of endotoxin hyporesponsiveness with TLR4 mutations and of a polymorphism in TLR2 gene with staphylococcal infection have been described in human (Arbour *et al.*, 2000; Lorenz *et al.*, 2000). Interestingly, this TLR2 missense mutation was shown to be associated with a subgroup of AD patients with an increased disease severity (Ahmad-Nejad *et al.*, 2004), suggesting a critical role of TLR2 and TLR4 in SEB recognition with respect to clinically relevant polymorphisms.

In the early steps of *S. aureus* infection, skin is not yet damaged by local inflammation, and exotoxins could more easily than the whole bacteria reach epidermal and dermal target cells expressing the appropriate TLR, as supported by detection of SEB in the dermis after epicutaneous application (Skov *et al.*, 2000). These first actors of innate sensing could include keratinocytes that constitutively express TLR2 but no TLR4 (Kollish *et al.*, 2005), epidermal Langerhans cells, and dermal DCs. We can make the assumption that interactions between SEB and TLR on those various cell types may specifically contribute to disease progression.

Finally, we observed significant differences in IL-12p70 secretion between monocytes from AD and non-atopic individuals in response to stimulation with LPS, as previously reported (Arbour *et al.*, 2000; Aiba *et al.*, 2003). Interestingly, our study showed differences between children and adult patients, since monocytes from some of the children tested produced the bioactive form of IL-12 after treatment with LPS. We could make the hypothesis that in these children, monocytes hyporesponsiveness was abrogated for reasons that remain to be determined but which could include a better ability to control *S. aureus* infection, due for instance to a protective TLR phenotype. Molecular mechanisms for endotoxin tolerance are not fully elucidated but could include reduction in TLR expression as well as modulation of intracellular signaling. Interestingly, synergy and cross-tolerance between TLR2 and TLR4 signaling have been described (Sato *et al.*, 2000), which could have sense in AD progression with respect to the ability of SEB to interact with both TLR.

Recovery of cell capacity to produce IL-12 upon inflammatory conditions could provide new clues to explain spontaneous disease regression in some children, whereas lack of this cytokine could favor disease persistence in adults.

Besides the well-known evidence that impaired recognition of *S. aureus* cell wall components by TLR2 may contribute to the pathogenesis of AD (Sato *et al.*, 2000), this report provides new insights into how bacterial toxins could take part in this process. Furthermore, a possible recognition of toxins by TLR2 and TLR4 on keratinocytes provides new clue on how colonization by *S. aureus* could impair expression of antimicrobial peptides such as human β -defensin-2. Finally, since the development of T_H1 or T_H2 response may be controlled by TLRs activation, blocking the binding of *S. aureus* toxins to these receptors could be considered as a new therapeutic approach.

MATERIALS AND METHODS

Donors

After informed consent, 30 patients with moderate to severe AD, aged from 6 month to 54 years, were enrolled (15 children under 14 years of age and 15 adults). Fifteen non-atopic adults were recruited as control. Disease severity was assessed using the SCORAD cumulative index (Anonymous, 1993). The study was approved by the Hospital Ethical Committee. The study was conducted according to the Declaration of Helsinki Principles.

Selection of CD14⁺ monocytes

Peripheral blood mononuclear cells from non-atopic or atopic individuals were isolated by standard density-gradient centrifugation with Ficoll-Paque (Amersham Pharmacia Biotech, GE Healthcare Europe GmbH, Orsay France) from 5 ml of heparinized blood.

CD14⁺ monocytes were isolated by magnetic cell sorting using positive selection with CD14⁺ microbeads (Miltenyi Biotech, Paris, France), according to the manufacturer's instructions. Isolated CD14⁺ monocytes (purity of 98%) were cultured in AIM-V serum-free medium (Life Technology, Invitrogen SARL, Cergy Pontoise, France) at a concentration of 1×10^6 cells ml⁻¹ at 37 °C in a humidified 5% CO₂ environment. Monocytes were left in culture before stimulation with either SEB (1–1,000 ng ml⁻¹; Sigma-Aldrich, L'isle d'Abeau Chenes, St Quentin Fallavier, France) or high pure LPS (1–1,000 ng ml⁻¹; InvivoGen, Cayla-invivogen Europe, Toulouse, France) for 24 hours. Alternatively, highly purified SEB was used as described (a gift from KR Acharya) (Mandron *et al.*, 2006). Cells were harvested for analysis of surface protein expression by cytofluorometric analysis, and supernatants were collected and stored at –20 °C for subsequent cytokines analysis.

Phenotypic analysis of monocytes

After 24 hours of stimulation, monocytes were harvested and washed with phosphate-buffered saline-bovine serum albumin (5%) and 10 mM EDTA. Then, they were incubated for 20 minutes with various fluorescent mAbs in phosphate-buffered saline-bovine serum albumin, washed in phosphate-buffered saline, and analyzed by flow cytometry using a Coulter Epics. Conjugated mAbs were as follows: FITC-anti-CD14 (RMO52) and FITC-anti-HLA-DR (B8-12-2) from Beckman-Coulter Immunotech (BC France SAS, Villepinte, France) PE-anti-CD54 (HA58), FITC-anti-TLR2 (TL2.1), and PE-anti-TLR4 (HTA125) from eBioscience (Clinisciences SA Montrouge, France) anti-TCR-PE (Miltenyi), and anti-CD56-PE (Beckman-Coulter Immunotech) to assess the presence of T cells and NK (natural killer) cells in monocytes preparations. Contamination never exceeded 1% for T cells and was less than 1% for NK cells.

Alternatively, CD14 sorted cells were incubated with increasing concentrations of recombinant IFN- γ (Roche, Neuilly sur Seine, France) as indicated and analyzed for HLA-DR expression by flow cytometry.

Analysis of cytokine secretion

Monocytes (1×10^6 cells ml⁻¹) were stimulated for 24 hours with 10 ng ml⁻¹ of LPS or SEB. Concentrations of cytokines in the culture supernatant were measured, as indicated, either by ELISA (Ready set Go, eBioscience) or by using Cytometric Bead Array (BD Biosciences, BD, Z.I des îles, Le Pont de Claix, France) according

to the manufacturer's instructions. Acquisition was performed on FACS Calibur (BD Biosciences). T cells and NK cells were purified by using anti-TCR- and anti-CD56-coated beads, respectively, according to Miltenyi Biotech's instructions. Cells were treated with SEB for 24 hours and cytokines (IFN- γ , TNF- α) measured by ELISA as indicated. Alternatively, purified monocytes were cocultured at various cell ratio with autologous purified T cells as indicated, coculture stimulated with SEB for 24 hours and cytokines (IFN- γ , TNF- α) measured by ELISA as indicated.

Neutralization assays

One hour before addition of SEB or LPS (10 ng ml⁻¹), monocytes were incubated for 30 minutes at room temperature with 20 μ g ml⁻¹ of TLR2 neutralizing antibody (TL2.1 eBioscience) or 30 minutes at 37 °C with 20 μ g ml⁻¹ anti-TLR4 antibody (HTA125, eBioscience). Alternatively, a mixture of both anti-TLR was used as well as with anti-MHC class II antibodies (at 10 μ g ml⁻¹; clone L243, BioLegend, Ozyme, St Quentin en Yvelines, France). After 24 hours of incubation, supernatants were collected and TNF- α secretion was measured using a human TNF- α Ready set Go ELISA kit (eBioscience), according to the manufacturer's instructions.

Statistical analysis

Mean values were compared by using the Student's *t*-test. All tests were considered significant for *P* < 0.01.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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