

ORIGINAL ARTICLE

Immunoglobulin E antibody reactivity to bacterial antigens in atopic dermatitis patients

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Summary

Background Atopic dermatitis (AD) is a chronic inflammatory skin disease affecting up to 20% children and 9% adults world-wide. AD patients are often sensitized against a broad variety of allergens and more than 90% of them suffer from skin superinfections with *Staphylococcus aureus*. **Objective** In this study, we searched for the presence of specific IgE antibodies against *S. aureus* and *Escherichia coli* antigens in AD patients.

Methods Sera from AD patients ($n = 79$), patients suffering only from allergic rhinoconjunctivitis ($n = 41$) or allergic asthma ($n = 37$) were tested for IgE reactivity to nitrocellulose-blotted *S. aureus*, *E. coli* and gut bacterial antigens. IgE-reactive bacterial antigens were affinity purified and identified by mass spectrometry.

Results More than 30% of AD patients but not patients suffering only from allergic rhinoconjunctivitis and asthma or non-allergic persons exhibited IgE binding to several protein antigens among them DNA-binding and ribosomal proteins and flagellin. Patients with severe skin manifestations showed more frequently IgE reactivity to *S. aureus* compared with AD patients with mild symptoms. Positive immediate and late skin test reactions could be induced in sensitized AD patients with *S. aureus* extract.

Conclusion and Clinical Relevance Specific IgE reactivities against a variety of bacterial antigens were observed in a subgroup comprising a third of AD patients and may contribute to allergic inflammation.

Keywords Atopic dermatitis, bacterial allergen, bacterial antigens, *Escherichia coli*, *Staphylococcus aureus*

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects up to 20.5% of children and between 0.2% and 8.8% of adults [1–4]. The clinical manifestations of AD vary and can range from dry skin and eczematous lesions to intense pruritus and lichenified flexures [5]. It has been reported that about 80% of AD patients exhibit elevated levels of serum IgE, and the IgE levels are often correlated with disease severity [6, 7]. As AD is associated with other atopic diseases such as asthma and allergic rhinitis, patients with AD often have specific IgE anti-

bodies and allergic symptoms to a great variety of food and inhalant allergens [8, 9].

Individuals suffering from AD show an increased susceptibility to cutaneous bacterial, viral and fungal infections [10, 11]. The predominant skin infection in AD is caused by *Staphylococcus aureus*, which affects between 29% and 100% of patients [12–14]. *S. aureus* is present at 100–1000-fold higher density (about 10^5 cfu/mL) in the skin of AD patients compared with the skin of healthy individuals [15]. In contrast, only 5–8% of healthy persons harbour *S. aureus*, which is usually concentrated in their mucosal cavities [16]. Density and frequency of

S. aureus colonization are significantly correlated with the severity of eczema [14, 17]. Furthermore, treatment of *S. aureus* skin infections with anti-staphylococcal antibiotics significantly reduces bacterial count and clinical severity of the disease [18, 19].

Escherichia coli is not a common microflora in infected AD lesions. In a study by Brook, *E. coli* was isolated from secondary infected eczema lesions of 10% of AD patients, and the colonization was restricted to the leg and buttock regions [12]. This was in contrast to *S. aureus*, which was detected in 29% of the patients in the same study, and was recovered from all body sites [12]. In another study, *E. coli* was isolated from the diaper area of between 0.3% and 1.1% of children with AD, which was much lower compared with *S. aureus* (4.2–10.8%) in the same study [20]. There have been no reports on the exacerbation of AD due to *E. coli* infection.

Beginning from the early 1980s, several groups reported that specific IgE against *S. aureus* proteins could be detected in the serum of AD patients [21–25]. Anti-*S. aureus* IgE titres were mostly observed in patients with moderate to severe AD [22, 24] but no detailed information about the IgE-reactive antigens was available except that both cellular proteins and cell wall components of *S. aureus* may be involved [24, 26–30]. Furthermore, some of the toxins were shown to react with IgE antibodies [31–33].

In the present study, the prevalence of serum IgE binding to antigens from *S. aureus* and *E. coli* was studied in patients suffering from AD of different severity, allergic rhinoconjunctivitis or allergic asthma by IgE immunoblotting. The nature of the IgE-reactive antigens was characterized by determination of their molecular weights, testing for anti-carbohydrate IgE reactivity and IgE inhibition experiments in different populations of AD patients. Additionally, effects of *S. aureus* and *E. coli* protein stimulation were evaluated by lymphoproliferations and measurements of cytokine secreted. IgE reactivity to proteins from seven most commonly occurring ileum and colon-colonizing bacteria were studied by immunoblotting. Furthermore, immune complexes consisting of IgE and bacterial antigens were affinity purified and subjected to mass spectrometry to identify IgE-reactive bacterial proteins. The allergenic activity of *S. aureus* antigens was investigated by skin testing in sensitized AD patients.

Methods

Characterization of patients

Sera from 35 Austrian and 44 German patients who according to the criteria of Hanifin and Rajka [34] suffered from AD were analysed. Tables 1 and 2 summarize the demographic, clinical and serological data of these patients. For control purposes, sera from Austrian patients with allergic rhino-conjunctivitis but no AD ($n = 41$) and

allergic asthma without AD ($n = 37$), and from nine non-atopic individuals were included. To investigate possible associations between severity of AD, *S. aureus* skin super-infections and IgE reactivity profiles, AD patients from Germany were tested. Serum samples were from patients who had undergone routine clinical testing and were used in an anonymous manner, with approval from the respective local ethics committees.

Preparation of bacterial total protein extracts

S. aureus (subspecies Rosenbach, ATCC 25923) and *E. coli* (strain Seattle 1946, ATCC 25922) were grown overnight in tryptic soy broth at 37 °C. The other bacterial species (Table S1) were cultured on trypticase soy agar plates with 5% sheep blood. All bacterial species used were well-characterized type cultures, purchased from either ATCC or the German collection of microorganisms and cell cultures (DSMZ). The bacterial cells were harvested by centrifugation at 3220×*g*. The cells were washed twice with phosphate-buffer saline (PBS), re-suspended in PBS and homogenized using an Ultra Turrax (IKA Labortechnik, Staufen, Germany) and glass beads. The homogenates were then centrifuged at 20 000×*g* for 10 min and the supernatants stored at –20 °C until use.

Immunoglobulin E immunoblotting, chemical deglycosylation, immunoglobulin E-immunoblot inhibitions

Bacterial extracts (*S. aureus*, *E. coli*) were boiled for 5 min with sodium dodecyl sulphate (SDS) sample buffer containing 5% v/v β-mercaptoethanol [35]. Approximately 200 µg/cm of each bacterial extract was separated on a 12.5% preparative SDS-PAGE [35]. A protein molecular weight marker (Rainbow-Marker, Amersham, Buckinghamshire, UK or PageRuler Prestained Protein Ladder Plus, Fermentas, St Leon-Rot, Germany) was used as a standard. The separated proteins were then transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by electroblotting [36]. Nitrocellulose strips were cut from the membranes and blocked twice for 10 min and once for 30 min in buffer A (50 mM sodium phosphate buffer, pH 7.4, containing 0.5% w/v bovine serum albumin (BSA), 0.5% v/v Tween 20 and 0.05% w/v NaN₃) at room temperature. Strips were incubated overnight with patients' sera (diluted 1 : 10 in buffer A) at 4 °C. The strips were then washed as described for blocking. Bound IgE was detected by incubating the strips with ¹²⁵I-labelled anti-human IgE antibodies (Pharmacia, Uppsala, Sweden) diluted 1 : 10 in buffer A, overnight at room temperature. After repeated washes in buffer A, the strips were dried and exposed to Kodak X-OMAT S films at –70 °C using intensifying screens (Kodak, Heidelberg, Germany).

Table 1. Demographic, clinical and serological characterization of AD patients from Austria*

Patient	Sex	Age	Symptoms	Allergies			Total IgE		SA IgE	EC IgE	SA enterotoxin RAST (RAST class)
				Pollen	Food	Other	(kU/L)				
A1	M	56	AD, RC	t, g, w		mi	1720	+	+	SEA (1), SEB (2), SEC (2), SED (1), SEE (2), TSST (2)	
A2	M	38	AD, RC	t, g, w	s, n, ap, c	a, mi	> 2000	+	+	SEA (2), SEB (2), SEC (2), SED (2), SEE (2), TSST (2)	
A3	M	39	AD, RC	t, g, w		mi	1060	—	+	SEA (0), SEB (0), SEC (0), TSST(1)	
A4	F	61	AD, AS	t, g	s	mi	ND	+	+	SEA (1), SEB (2), SEC (2), SED (1), SEE (1), TSST (1)	
A5	F	45	AD	t, g	mk	a, mi	ND	+	—	ND	
A6	F	80	AD, AS	t, g		a	> 1000	+	+	SEB (1), SEC (1), TSST (2)	
A7	F	44	AD		ap, n		165	—	—	SEA (0), SEB (2), SEC (0), TSST (0)	
A8	F	46	AD		c	a, mi	271	—	—	SEA (0), SEB (0), SEC (0), TSST (0)	
A9	F	43	AD, RC	t, g	ap, n	a	802	—	—	SEA (0), SEB (2), SEC (0), TSST (1)	
A10	F	73	AD	g		mi	> 2000	+	—	SEA (2), SEB (2), SEC (2), SED (2), SEE (2), TSST (3)	
A12	M	52	AD, RC, AS			a, mi	> 2000	+	+	SEA (2), SEB (2), SEC (2), SED (2), SEE (2), TSST (2)	
A13	M	67	AD			mi	> 5000	—	+	SEA (1), SEB (2), SEC (2)	
A14	F	29	AD, RC	t, g, w		mi	46	—	—	SEA (0), SEB (0), SEC (0)	
A15	F	36	AD, AS	t, g, w		a, mi	580	—	—	ND	
A16	F	38	AD, RC	t, g, w		a	270	—	—	SEA (0), SEB (0), SEC (0)	
A17	M	66	AD, RC	t, g, w	n, ap	mi	932	—	—	ND	
A18	M	34	AD, RC	t, g, w			> 2000	—	+	ND	
A19	F	37	AD, RC	t, g, w	n, ap, k		339	—	—	ND	
A20	M	36	AD, RC	t, g, w	ap, ca		> 2000	—	—	SEA (2), SEB (1), SEC (1), SED (1), SEE (1), TSST (2)	
A21	M	48	AD, RC, AS	t, g, w	n, ap, k, ca	mi	> 2000	—	+	TSST (2)	
A22	F	54	AD, RC, AS	t	ap, ca, c		187	—	—	SEA (0), SEB (0), SEC (0)	
A23	M	49	AD, RC, AS	g		mi	196	—	—	ND	
A24	F	34	AD, RC	t	n, ap, c	a	189	—	—	SEA (0), SEB (0), SEC (0)	
A25	F	29	AD, RC, AS	t, g, w		mi	294	—	—	ND	
A26	M	37	AD, RC	t, g, w			380	—	—	ND	
A27	M	41	AD, RC	t, g, w		a, mi	290	—	—	SEA (0), SEB (0), SEC (0)	
A28	M	34	AD, RC	t, g, w	ap	a	309	—	—	ND	
A29	M	36	AD, RC	t, g, w	n, ap, k		512	—	—	ND	
A30	M	51	AD, RC	t, g, w	k	mi	80	—	—	ND	
A31	M	38	AD, RC	t			27	—	—	ND	
A32	F	34	AD, RC, AS	g	n, k, ca		112	—	—	ND	
A33	F	30	AD, RC	t, g, w		a, mi	> 2000	+	+	SEA (1), SEB (2), SEC (3), SED (2), TSST (2)	
A34	M	27	AD	t, g, w	n, ap, p	a	205	—	—	ND	
A35	F	43	AD, RC	t, g	n, ap, p, mk	a, mi	19464	+	+	ND	
A36	F	31	AD	t	ap	a	2923	+	+	ND	

*a, animals; AD, atopic dermatitis; ap, apple; AS, asthma; c, celery; ca, carrot; EC, *E. coli*; F, female; g, grass; k, kiwi; kU/L, kilo units per liter; M, male; mi, mites; mk, milk; n, nuts; ND, not determined; p, peach; RC, rhinoconjunctivitis; s, seafood; SA, *S. aureus*; SE, staphylococcal enterotoxins; t, trees; TSST, toxic shock syndrome toxin; w, weeds; +, positive reaction; —, negative reaction.

For chemical deglycosylation, nitrocellulose-blotted bacterial extracts were incubated with 50 mM sodium acetate, pH 4.5 with or without 10 mM periodate for 2 h at 4 °C. Strips were washed twice for 10 min and once for 30 min with buffer A, and were then incubated with patients' sera diluted 1 : 10 in buffer A overnight at 4 °C. Bound IgE antibodies were detected as described for immunoblotting. For inhibition experiments, patients' sera (diluted 1 : 10 in buffer A) were pre-incubated with bacterial extracts overnight at 4 °C (1000 µg bacterial extract per 1 mL of diluted sera). The nitrocellulose-blotted bacterial extracts were then incubated with the pre-adsorbed sera overnight at 4 °C, and bound IgE antibodies were detected as described for immunoblotting.

Measurement of *Staphylococcus aureus* enterotoxin-specific immunoglobulin E antibodies

Serum IgE antibodies against *Staphylococcus* enterotoxin (SE) A, SEB, SEC, SED, SEE and toxic shock syndrome toxin (Toxin Technology, FL, USA) were measured using the CAP assay (Phadia, Uppsala, Sweden) [37].

Specific lymphocyte proliferation and cytokine responses to *Staphylococcus aureus* and *Escherichia coli* protein extracts

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood from four AD and five non-allergic

Table 2. Demographic, clinical and serological characterization of AD patients from Germany*

Patient	Sex	Age	Symptoms	SCORAD	Allergies			Total IgE			Bacterial infection
					Pollen	Food	Other	(kU/L)	SA IgE	EC IgE	
H1	M	35	AD, AS, AR	45	t, g, w	so	a, f, mi, asp	12499	+	—	N
H3	M	32	AD, AS, AR	44	t			68.7	—	—	N
H4	F	40	AD, AR	24	t, g, w		a, f, mi	1119	—	—	N
H6	M	72	AD	33	t, g, w		a, f, mi, asp	3668	+	+	N
H8	F	25	AD, AS, AR	42	t, g, w		a, f, mi, asp	1498	—	—	N
H9	F	47	AD, AS, AR	31	t, g, w		a, mi, asp	1020	—	+	N
H10	M	25	AD, AS, AR	31	t, g, w		a, f, mi, asp	2262	—	+	Y
H12	F	22	AD, AS, AR	61	t, g, w	n, so, mk, s, wh	a, f, mi, asp	66286	+	+	Y
H13	F	27	AD	22			mi	36	—	—	N
H14	F	29	AD	12	ND	ND	ND	38.5	—	—	N
H15	F	29	AD, AS, AR	13	ND	ND	ND	43.9	—	—	N
H16	M	22	AD, AR	16	t, g		a, mi	1308	—	—	N
H17	M	40	AD	28			f	2298	—	—	N
H18	F	37	AD, AS, AR	51	t, g, w	n, so	a, mi, asp	26200	+	+	N
H20	M	48	AD	30			mi	184	—	—	N
H21	F	34	AD	24			a, f, mi	1310	—	—	N
H23	F	26	AD	52	w		a, f, asp	843	—	+	Y
H24	F	38	AD	51	t, g		a, f, mi, asp	2661	—	—	Y
H26	M	20	AD, AR	54	g		a, f, mi, asp	5643	+	—	Y
H27	M	30	AD, AS, AR	20	t, g, w	so	a, f	1042	—	+	N
H28	M	43	AD, AS, AR	60	t, g, w	e, s, wh, n, so	a, f, mi, asp	15119	+	+	N
H29	M	62	AD	56	t, g, w	wh, n, so	a, f, mi, asp	11544	+	+	N
H30	M	49	AD, AS	46	t, g, w		a, f, mi, asp	29240	+	+	N
H32	F	28	AD, AS, AR	50	t, g, w		a, mi	1815	—	—	Y
H34	F	46	AD, AS, AR	53	t, g, w		a, f, mi, asp	11403	+	—	N
H35	M	60	AD, AS, AR	41			a, mi, asp	12841	+	+	N
H36	F	19	AD	48			a, f, asp	830	—	—	Y
H37	F	23	AD, AR	10	t, g		a, mi	861	—	—	N
H38	M	37	AD	22	ND	ND	ND	13.8	—	—	N
H39	M	25	AD, AR	31	t, g, w		a, f, mi, asp	3964	+	—	N
H40	M	32	AD, AS	25	t, g, w		a, f, mi, asp	13113	+	+	Y
H42	F	33	AD, AS, AR	61	t, g	so	f, mi	1216	—	—	N
H43	F	20	AD	54	ND	ND	ND	5.9	—	—	Y
H45	F	44	AD	47	ND	ND	ND	23.4	—	—	N
H46	M	36	AD, AS	49	t, g, w	k, s, e, mk, wh, n, so	a, f, mi, asp	19790	+	+	N
H47	M	44	AD, AS, AR	43	t, g, w		a, f, mi, asp	19545	+	—	N
H48	M	53	AD, AS	62	t, g, w		a, f, mi, asp	21428	+	+	N
H49	M	34	AD, AS, AR	56	t, g, w		a, f, mi, asp	33700	+	+	N
H50	F	18	AD, AS, AR	28			mi	31.5	—	—	N
H51	F	39	AD, AS, AR	47	t, g	n	a, f, mi, asp, l	463	—	—	N
H54	F	26	AD, AS, AR	8	t		f	68.5	—	—	N
H55	M	43	AD, AR	49	t, g, w		a, f, mi, asp, l	11950	+	+	N
H56	F	54	AD, AS, AR	45	g, w		a, f, mi, asp, l	9400	+	+	N
H57	M	25	AD, AS, AR	63	t, g, w		a, f, mi, asp, l	40200	+	+	N

*a, animals; AD, atopic dermatitis; AS, asthma; asp, *Aspergillus fumigatus*; AR, allergic rhinitis; e, egg; EC, *E. coli*; f, fungus; F, female; g, grass; k, kiwi; kU/L, kilo units per liter; l, latex; M, male; mi, mites; mk, milk; N, no; n, nuts; ND, not determined; s, seafood; SA, *S. aureus*; so, soy; t, trees; w, weeds; wh, wheat; Y, yes; +, positive reaction; —, negative reaction.

individuals using Ficoll-Paque gradient centrifugation and were resuspended in Ultraculture medium (Bio Whittaker, Walkersville, MD, USA) supplemented with 2 mM glutamine, 50 mM β -mecarptoethanol and 0.1 mg/mL gentamycin. Cells were cultured in sterile 96-well plates (Nunc) at 2×10^5 cells/well and stimulated with *S. aureus* extract

(50 μ g/well), *E. coli* extract (50 μ g/well), IL-2 (positive control) or medium (negative control) in triplicates at 37 °C with 5% CO₂. Six days after initial stimulation, 0.5 μ Ci of ³H-thymidine (Amersham) was added to each well, and cells were incubated for 16 h. ³H-thymidine incorporation was measured by scintillation counting (Microbeta

Trilux, Perkin Elmer, Waltham, MA, USA) and are reported as stimulation index, calculated as the ratio of the mean proliferation after antigen stimulation over medium control values. Cytokine levels (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, GM-CSF, TNF- α and IFN- γ) were measured in supernatants collected from PBMC cultures at day 6, using the Bio-Plex Pro Human Cytokine TH1/TH2 assay kit (Bio-Rad, Hercules, CA, USA) and the assay was performed according to the manufacturer's instructions and fluorescent signals were read on a Bio-Plex system (Bio-Rad).

Skin testing

Routine skin testing was performed by intradermal injection of aliquots of 20 μ L of *S. aureus* extract, histamine or saline into forearms of 22 AD patients in the Russian centre. Patients had to be in remission regarding AD and pyoderma and did not use any pharmacotherapy for at least 5 days before skin testing. Skin reactivities were recorded after 20 min and 24 h.

Identification of immunoglobulin E-reactive *Staphylococcus aureus* and *Escherichia coli* proteins by mass spectrometry

An anti-human IgE column was prepared by coupling 1.5 mg of monoclonal mouse anti-human IgE (BD Pharmingen, San Diego, CA, USA) on a HiTrap NHS-activated HP column (GE Healthcare, Uppsala, Sweden). The column was washed with PBS to remove unbound antibodies and loaded with serum IgE of AD patients. Then the column was washed with PBS and loaded with *S. aureus* or *E. coli* extract at a concentration of 1 mg/mL. Unbound proteins were removed by washing with PBS and IgE immune complexes were eluted with 0.1 M glycine pH 2.8, and neutralized immediately with 1 M Tris pH 10. The eluted fraction was desalted using a PD10 column with a solution of 5 mM NaH₂PO₄, pH 7.0. Eluted proteins were then digested overnight at 37 °C with trypsin (Sigma, St. Louis, MO, USA), and subjected to mass spectrometry on Nano-LC system coupled to Bruker HCT Ultra ESI trap (Bruker Daltonics, Bremen, Germany). The peptides were fractionated on a C18 PepMap 100 column (3 μ m, 100 Å) (LC Packings, Dionex, Sunnyvale, CA, USA) using a 5%–80% acetonitrile solvent gradient. During elution, the mass spectra were recorded in the mass/charge range of 300–3000. Data were processed using DataAnalysisTM 3.4 (Bruker Daltonics, Bremen, Germany), and the peak list generated was searched against the Swiss-Prot database subset of eubacteria proteins using MASCOT (Matrix Science) search engine. The search setting included a missed cleavage site value of three, carboxymethylation of cysteine and variable oxidation of methionine, histidine and tryptophan. Protein matches with scores greater than 50 indicated relevant identity to known proteins ($P < 0.05$).

Results

A third of atopic dermatitis patients exhibit specific immunoglobulin E reactivity to various protein antigens from Staphylococcus aureus and Escherichia coli

IgE reactivities to nitrocellulose-blotted *S. aureus* and *E. coli* antigens (Figs 1 and 2) were analysed using sera from 35 AD patients from Austria (Table 1) and 44 AD patients from Germany (Table 2). From the total of 79 patients, 39 were male, and the mean age of the patients was 39.3 years (range 18–80 years). The majority of patients also presented allergic manifestations other than AD, such as rhinitis and bronchial asthma, and were sensitized to multiple allergen sources. The patients displayed a wide range of total serum IgE levels (5.9–66286 kU/L).

Ten of the 35 AD patients from the Austrian centre showed distinct IgE reactivities to bands of different molecular weight (10–70 kDa) in extracts of *S. aureus* (Fig. 1a). Elevated total serum IgE levels were often associated with IgE reactivity to *S. aureus* but were not a pre-requisite for IgE binding. In fact, not all patients with high total serum IgE (e.g., A13, A18, A20, A21) contained *S. aureus*-specific IgE (Table 1; Fig. 1a). Similar results were obtained for the German AD patients (Table 2, Fig. 2a), where some patients (e.g. H10, H17, H32) with high total IgE levels showed no *S. aureus*-specific IgE. When the serum from a non-atopic individual was used (panel N, Figs 1a and 2a), no IgE binding was observed.

The same sera were also probed with nitrocellulose-blotted *E. coli* extract (Fig. 1B). Twelve of the 35 Austrian patients showed IgE reactivity to at least one band in *E. coli* extracts. Some of the IgE-reactive bands in *E. coli* extracts had similar molecular weights as those in *S. aureus* whereas others were different. Interestingly, certain patients showed IgE reactivity only to *E. coli* antigens (e.g., A13, A18, A21) but not to *S. aureus* antigens. Similarly in the German population, some patients (e.g., H9 and H27) had specific IgE reactivity only to *E. coli*, but not *S. aureus* proteins (Fig. 2b). Again IgE reactivity was often associated with high total serum IgE but there were also patients with high total serum IgE lacking IgE reactivity to *E. coli* antigens (e.g., A20, H1, H26, H47). A slight anti-IgE-reactive band at approximately 34–35 kDa was observed with all sera, including the non-allergic persons (panel N) serum as well as the buffer control (data not shown). Hence, it was a result of binding of the anti-IgE conjugate to this band. For control purposes, sera from 37 individuals with asthma and from 41 individuals with rhino-conjunctivitis but without allergic skin manifestations were also tested but no IgE reactivity to bacterial extracts was observed with these sera (data not shown).

Nineteen sera from the 35 AD patients were also tested for IgE reactivity to *S. aureus* toxins, of which 13 contained toxin-specific IgE (Table 1). Again toxin-

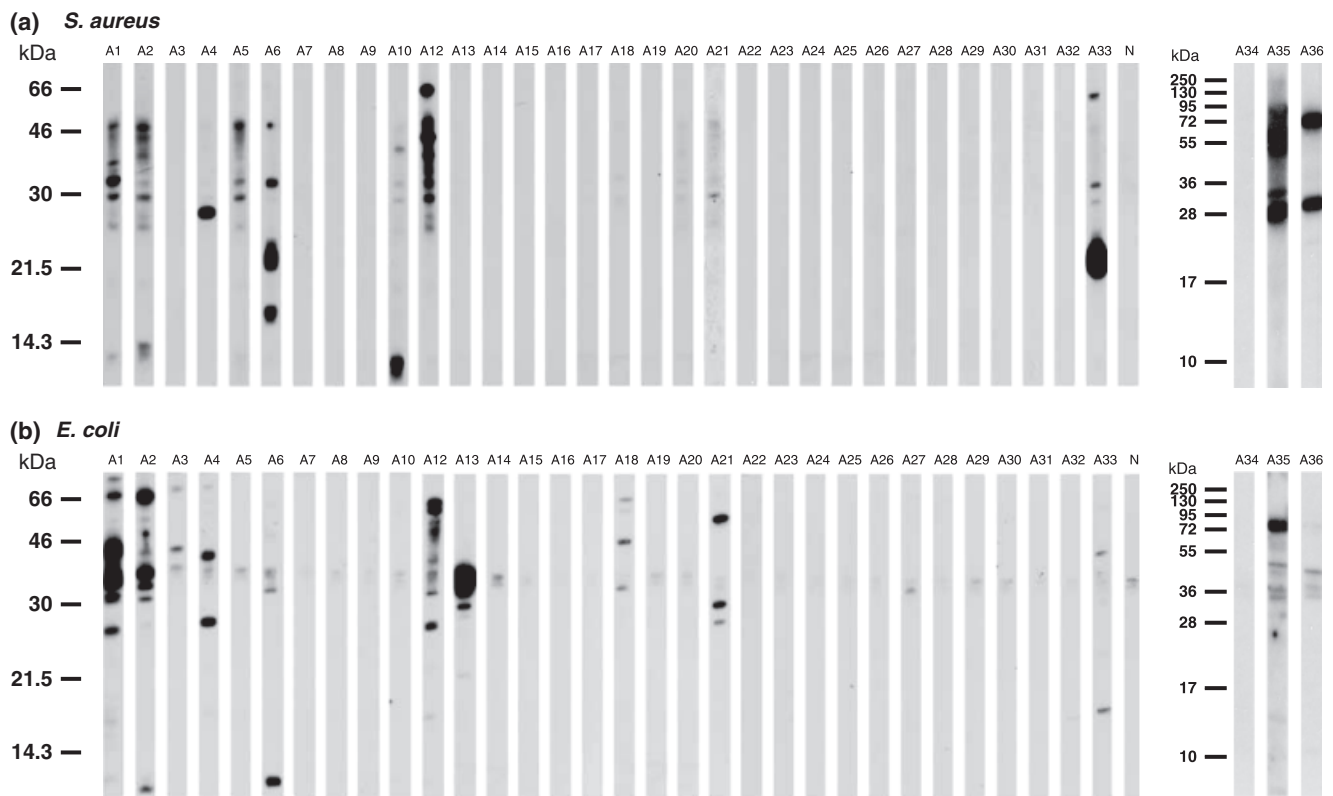


Fig. 1. IgE reactivity of AD patients to nitrocellulose-blotted *Staphylococcus aureus* and *Escherichia coli* protein extracts. Total protein extracts of *S. aureus* (a) and *E. coli* (b) were separated under reducing conditions by sodium dodecyl sulphate-polyacryl amide gel electrophoresis (SDS-PAGE), blotted on to nitrocellulose membrane and probed with sera from 35 patients with atopic dermatitis. As a control, serum from a non-atopic individual (panel N) was used. Molecular weights (kDa) are indicated in the left margin.

specific IgE was mainly found in sera containing high total serum IgE levels but there were also sera (e.g., A7) that contained low total IgE levels and toxin-specific IgE (Table 1).

Periodate deglycosylation assay was used to determine if the IgE-reactive antigens were proteins or carbohydrates. Pre-treatment of nitrocellulose-blotted *S. aureus* antigens with periodate did not lead to a relevant reduction of IgE-binding capacity of the bacterial antigenic structures, compared with treatment with buffer alone. Periodate treatment of *E. coli* antigens also did not show any change in IgE binding capacity in five of the six patients tested. In one patient, periodate treatment even revealed additional IgE-reactive bands of approximately 25 and 20 kDa (data not shown).

Patients with severe dermatitis symptoms show more frequently immunoglobulin E reactivity to bacterial antigens than patients with mild symptoms

Based on the disease severity score of AD (SCORAD) for the German AD patients (Table 2), we classified these patients as having mild (0–24), moderate (25–50) or severe (>50) disease. Interestingly, 0%, 48% and 77% of these

patients showed positive *S. aureus* IgE reactivity in the mild, moderate and severe groups, respectively. A similar relationship between *E. coli*-IgE reactivity and SCORAD was observed in this population (i.e. 10%, 48% and 77% for mild, moderate and severe AD, respectively). Hence, AD patients with an increased disease severity showed more frequently specific IgE reactivity to *S. aureus* and *E. coli* proteins, compared with AD patients with mild symptoms.

Next, we were interested to investigate if there was any association between *S. aureus* skin superinfections and IgE reactivity to bacterial antigens. For this purpose, sera from AD populations with and without skin superinfections (defined as clinically visible bacterial infection) obtained from the German centre were studied (Fig. 2). IgE reactivity to *S. aureus* antigens was observed in 33% (3 of 9) of patients with skin superinfections, and 43% (15 of 35) of patients without skin superinfections (Fig. 2a). Similarly, 44% (4 of 9) and 43% (15 of 35) patients had IgE reactivity to *E. coli* proteins in this group of patients (Fig. 2b). Hence, the frequency of IgE reactivity to bacterial antigens was not related to the presence of skin superinfections in AD patients.

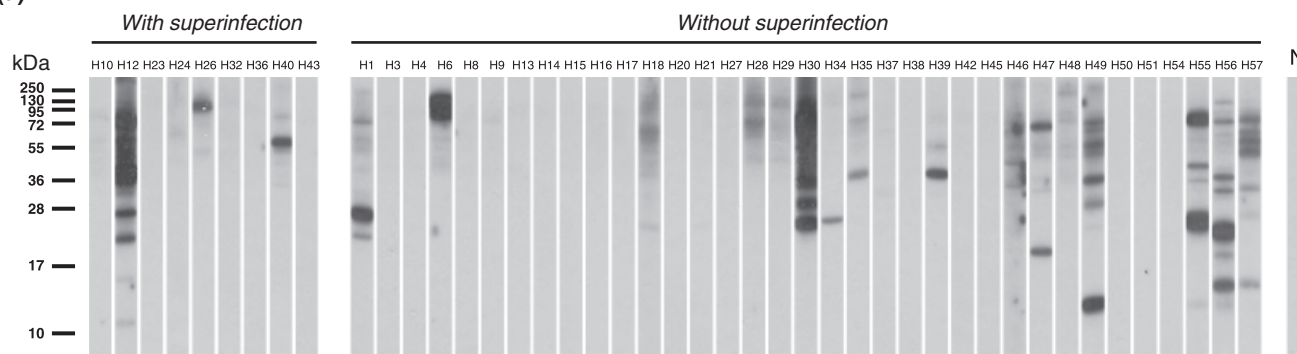
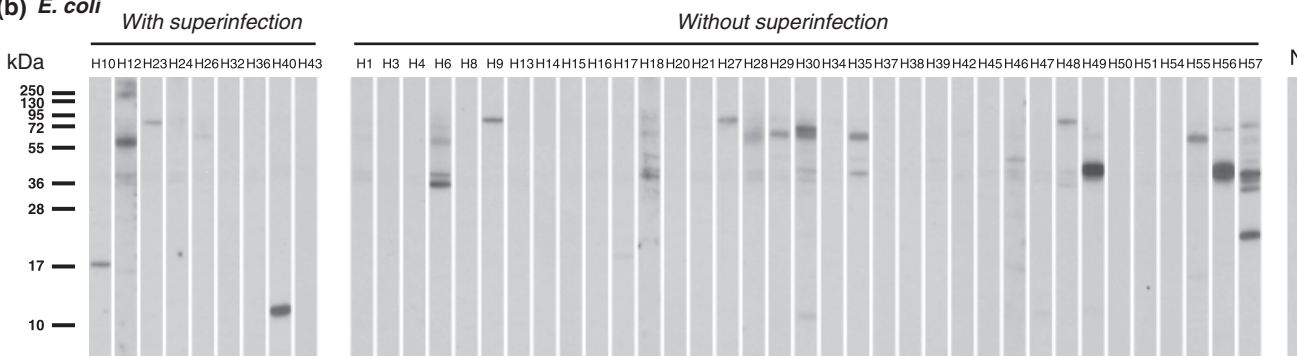
(a) *S. aureus***(b) *E. coli***

Fig. 2. IgE reactivity of atopic dermatitis patients with and without skin superinfections to nitrocellulose-blotted *Staphylococcus aureus* and *Escherichia coli* protein extracts. Total protein extracts of *S. aureus* (a) and *E. coli* (b) were separated under reducing conditions by SDS-PAGE, blotted on to nitrocellulose membrane and probed with sera from the German atopic dermatitis patient populations with or without skin superinfections. As a control, serum from a non-atopic individual (panel N) was used. Molecular weights (kDa) are indicated in the left margin.

Immunoglobulin E reactivity to gut bacteria

In this study, more than a third of the AD patients showed IgE reactivity to *E. coli* proteins. The route of *E. coli* sensitization occurs most likely from the gut, and we therefore studied if AD patients also show IgE binding to antigens of other gut bacterial species. Nitrocellulose-blotted protein extracts of seven of the most commonly occurring gut bacterial species of the ileum or colon region (Table S1) [38] were tested with remaining serum samples from AD patients with and without IgE-reactivity to *E. coli* proteins (Fig. 3a and b). We found that both groups of AD patients showed IgE reactivity to ileum- and colon-colonizing bacteria (Fig. 3a and b).

Low immunoglobulin E cross-reactivity between *Staphylococcus aureus* and *Escherichia coli* antigens

S. aureus and *E. coli* extracts contained IgE-reactive bands of similar molecular weights. We therefore searched for the presence of IgE cross-reactive structures in *S. aureus* and *E. coli* in patients who displayed IgE reactivity to both extracts and for whom sufficient serum was available (Fig. 4).

As shown in Fig. 4a, we found no (e.g., A4, A6) or limited cross-reactivity (e.g., A36, H12, H40) to nitrocel-

lulose-blotted *S. aureus* extract when patient's sera were pre-incubated with *E. coli* extract. Three of the four patients' sera (A4, A21 and H49) showed no IgE cross-reactivity between *S. aureus* extract on nitrocellulose-blotted *E. coli* proteins. Serum from patient H48 showed only limited cross-reactivity, to a protein band of approximately 32 kDa (Fig. 4b).

Lymphoproliferation and cytokine responses of atopic dermatitis patients to bacterial antigens

We found that *S. aureus* but not *E. coli* induced lymphoproliferative responses in AD as well as in non-allergic persons (Fig. 5a). We also measured the levels of cytokines released into the cell culture supernatants after stimulation with bacterial extracts (Fig. 5b). The stimulation of lymphocytes with proteins from *S. aureus* resulted in the release of elevated levels TH1 and TH2 cytokines, when compared with stimulation with *E. coli* extract or medium alone.

Staphylococcus aureus antigens can induce immediate-type skin reactions in sensitized atopic dermatitis patients

Figure 6 shows the immediate and late skin reactions of 22 patients to *S. aureus* extract. All but one of the 19 patients

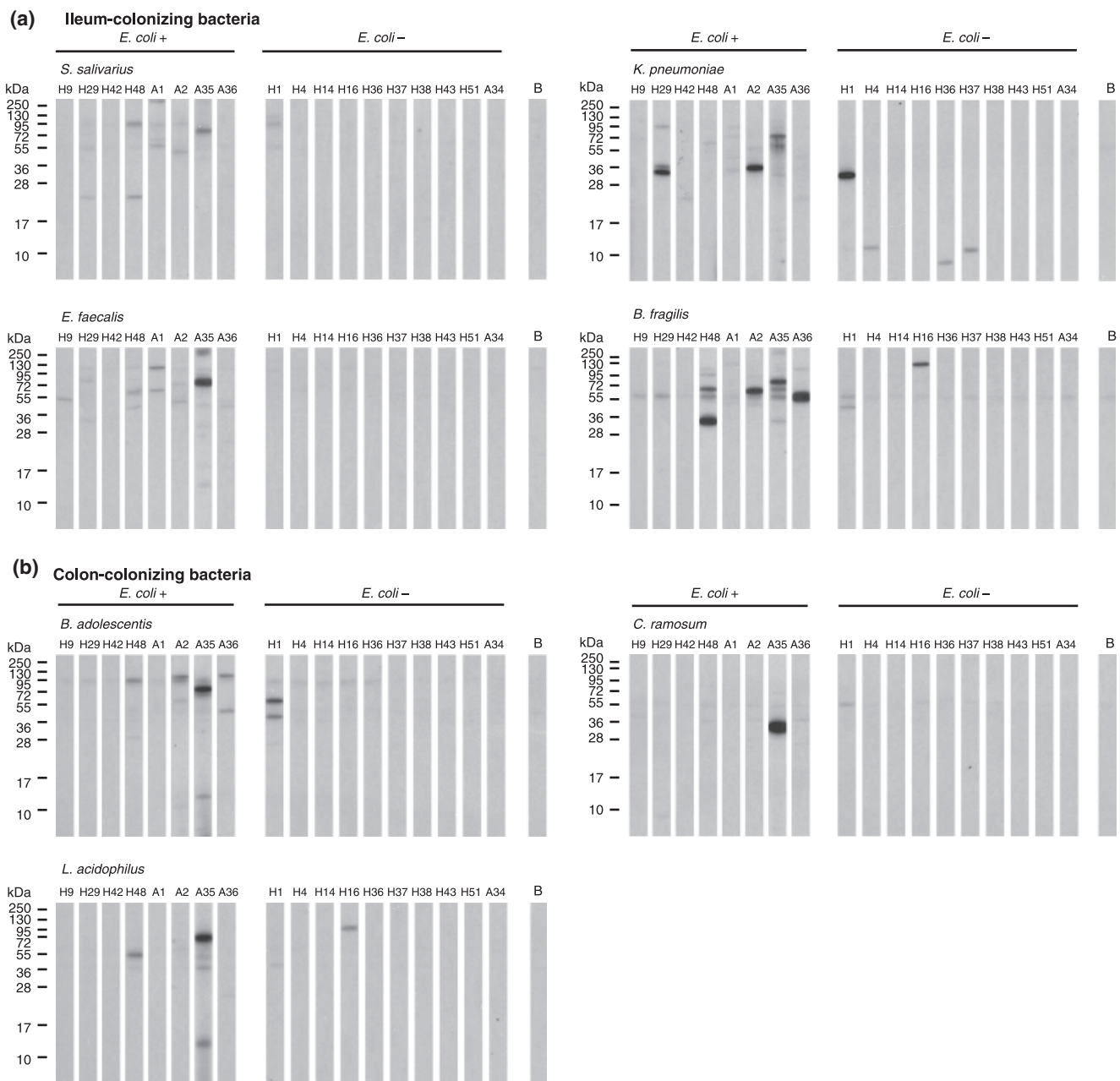


Fig. 3. IgE reactivity of atopic dermatitis (AD) patients to nitrocellulose-blotted protein extracts from gut-colonizing bacteria. Nitrocellulose-blotted protein extracts of *S. salivarius*, *K. pneumoniae*, *E. faecalis* and *B. fragilis*, which are ileum-colonizing bacteria (a) and *B. adolescentis*, *C. ramosum* and *L. acidophilus*, which are colon-colonizing bacteria (b) were probed with sera from patients with AD, with IgE *E. coli* + or without (*E. coli* -) to *E. coli* proteins. Lane B denotes buffer control. Patient numbers are as in Tables 1 and 2. Molecular weights (kDa) are indicated in the left margin.

with IgE antibodies to *S. aureus* proteins had both immediate and late phase inflammations upon intradermal skin testing with *S. aureus* extract. None of the patients who did not mount IgE against *S. aureus* IgE showed any skin reaction. The results show that *S. aureus* extracts can induce both specific immediate and late phase skin inflammation in patients with IgE antibodies against *S. aureus* components.

Identification of immunoglobulin E-reactive bacterial antigens by mass spectrometry

In order to identify IgE-reactive bacterial antigens, serum IgE from AD patients reacting with bacterial antigens was specifically immobilized on an anti-IgE column and incubated with bacterial antigens. Immune complexes consisting of IgE and bacterial antigens were then

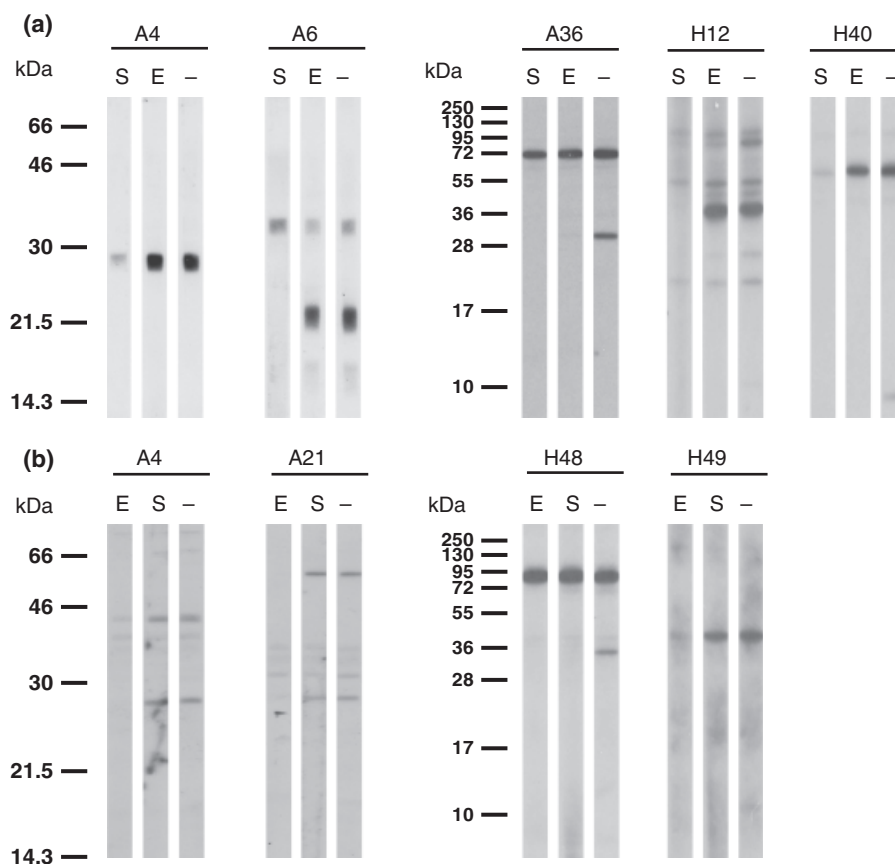


Fig. 4. Low cross-reactivity between IgE-reactive antigens from *Staphylococcus aureus* and *Escherichia coli*. Nitrocellulose-blotted *S. aureus* extracts (a) and *E. coli* extracts (b) were probed with sera from atopic dermatitis patients, which were pre-adsorbed with protein extracts of either *S. aureus* (lanes S), *E. coli* (lanes E), or with bovine serum albumin (BSA; lanes -). Patient numbers are as in Tables 1 and 2. Molecular weights (kDa) are indicated in the left margin.

purified and subjected to MS/MS analysis. Table S2 displays proteins from *S. aureus* and *E. coli* for which matching peptides could be identified by mass spectrometry in immune complexes obtained from four reactive AD patients with a probability of >0.05 . These proteins included among other proteins, DNA-binding proteins, ribosomal proteins and flagellin.

Discussion

In this study, we report that a subgroup comprising more than a third of AD patients exhibits specific IgE reactivity against a variety of protein antigens of diverse molecular weights in *S. aureus* and *E. coli*. IgE binding was directed to protein bands in the molecular weight range of 20–100 kDa, with a high IgE recognition frequency to antigens in the 30–66 kDa range. The specificity of the IgE reactivity to the bacterial antigens was underlined by the fact that there was no clear relationship between total serum IgE and the presence of specific IgE to antigens of *S. aureus* or *E. coli*. Elevated total serum IgE was associated

with IgE reactivity to bacterial antigens, but was not a prerequisite for this reaction. For the majority of patients, no or limited IgE cross-reactivity between antigens of *S. aureus* and *E. coli* was observed.

IgE reactivity to *S. aureus* has already been reported in earlier studies, but the antigens have not been characterized. We found that approximately 30% of AD patients had IgE against *S. aureus* antigens, whereas in earlier studies the prevalence of anti-*S. aureus* IgE in AD patients showed a great variation from 7% to 70%. This variation might be explained by the antigen preparation (whole cells [21, 25, 27, 39], protein extracts [23, 26] or purified components [24–27, 29, 30]), choice of detection systems (RAST [21–27, 30, 39], immunoblots [30], ELISA [29]) and also the patient population.

We investigated if the antigens of *S. aureus* and *E. coli* contain protein or carbohydrate epitopes. Based on the results of our chemical deglycosylation study, we did not detect a significant difference between IgE reactivity to periodate treated or untreated nitrocellulose-blotted antigens from *S. aureus* and *E. coli*, and hence conclude that

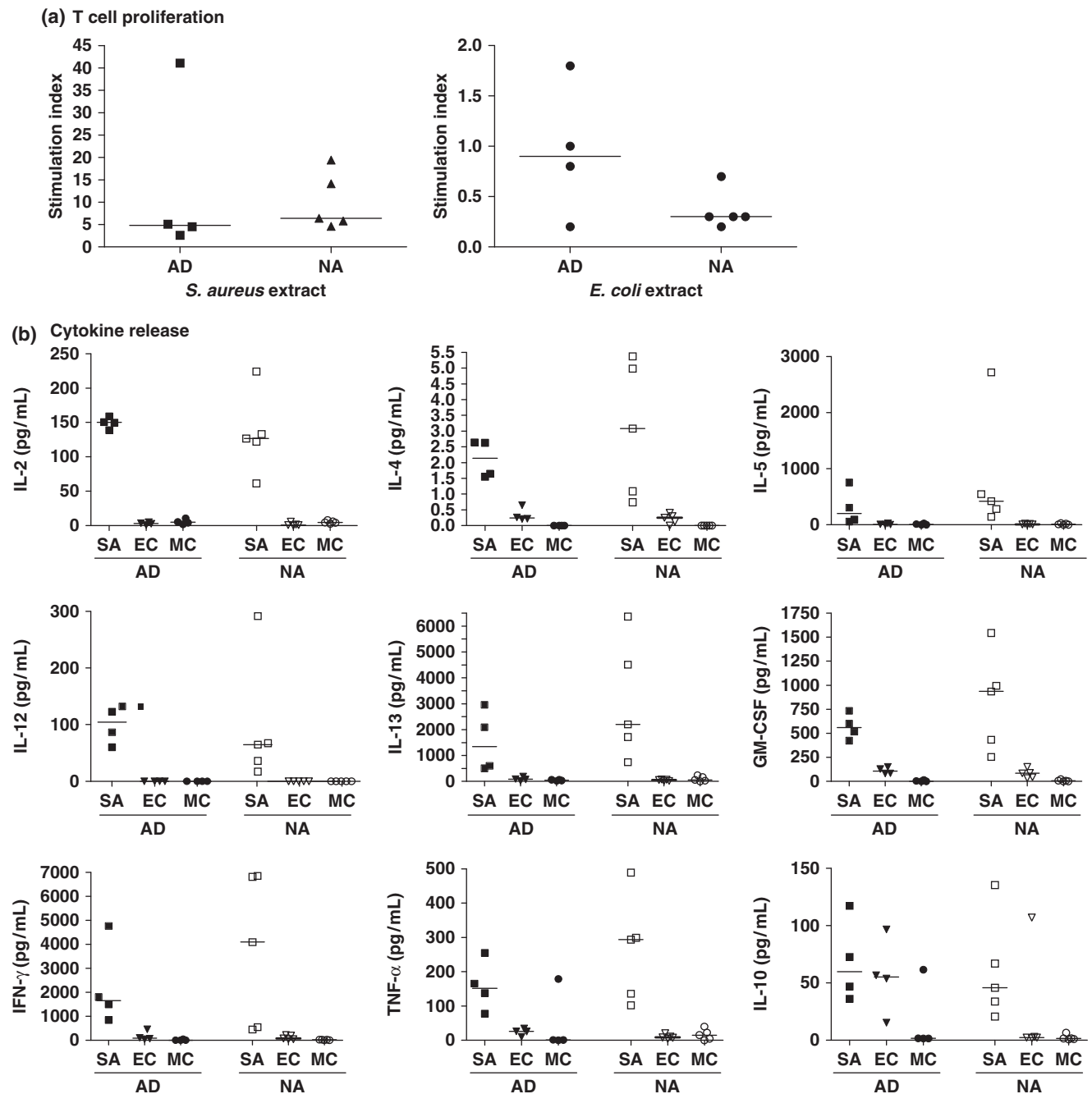


Fig. 5. Lymphocyte proliferation and cytokine releases induced in PBMC cultures of AD patients and non-allergic individuals by stimulation with *Staphylococcus aureus* and *Escherichia coli* protein extracts. (a) PBMCs from AD or non-atopic (NA) individuals were stimulated with *S. aureus* extract, *E. coli* extract or medium (MC). Mean lymphoproliferative responses of triplicate determinations were measured by ^3H -thymidine incorporation and are displayed as stimulation indices. Horizontal lines denote the median value of each group. (b) The amounts of released cytokines are presented and horizontal lines denote the median value of each group.

the bacterial antigens comprise mainly protein moieties. In fact, the analysis of IgE-reactive bacterial proteins by mass spectrometry identified several of these proteins, among them DNA-binding proteins, ribosomal proteins and flagellin.

A quite unexpected finding of this study was the presence of anti-*E. coli* IgE reactivity. Up to 38% of the AD patients

tested had specific IgE antibodies to *E. coli* antigens, although *E. coli* is not a common microflora in the infected skin lesions of AD patients. Based on the inhibition immunoblot assay, the IgE antibodies to *E. coli* proteins were mainly not cross-reactive to *S. aureus* antigens. With regard to the route of sensitization for *E. coli*, it is possible that it occurs via the skin, although there is little colonization.

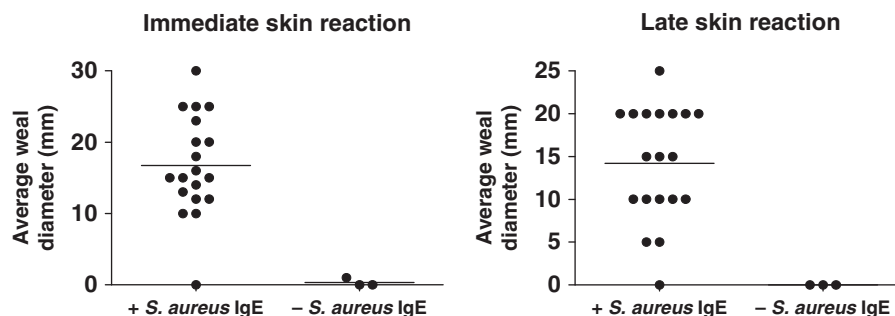


Fig. 6. Immediate and late type skin reactions to *Staphylococcus aureus* extract in AD patients. Twenty-two patients with AD, with or without IgE-reactivity to *S. aureus* proteins, were injected intradermally with *S. aureus* extract or saline buffer. Average weal diameter was measured 20 min (immediate reaction) and 24 h (late reaction) after the challenge.

Alternatively, the gut may be a more likely entrance and sensitization point for *E. coli*. Therefore, IgE reactivity to protein extracts of commonly occurring gut bacterial species of the ileum and colon regions was investigated and IgE binding to gut bacterial proteins was detected. Based on these results, it may be interesting to investigate in a clinical study whether IgE antibodies against *E. coli* can induce food allergy-like symptoms.

In the case of *S. aureus*, the most probable route of sensitization is the skin. In fact, we observed that patients suffering from severe forms of AD and with higher SCORAD values showed more frequently IgE reactivity to bacterial antigens compared with patients with mild AD symptoms and it is thus possible that patients with more severe skin damage experience higher loads of bacterial antigen exposure capable of boosting anti-bacterial IgE. The production of anti-microbial peptides, such as β -defensins and cathelicidins is reduced in AD patients compared with healthy persons [40, 41]. Additionally, it is known that many individuals with AD also have a defective skin barrier, and the combination of these two factors may enhance increased colonization of bacteria on the diseased skin, leading to an increased penetration of *S. aureus* antigens through the skin [42] and elevated specific IgE production.

Specific anti-*S. aureus* IgE was detected exclusively in patients with AD, but not in those suffering from other clinical manifestations of allergy, such as allergic asthma and rhino-conjunctivitis. This was also the case for another microorganism that was found to colonize the skin of AD patients, *Malassezia sympodialis* [40]. This finding could be a result of the continuous sensitizations resulting from the lack of innate immune function and disrupted skin barrier in AD patients. In contrast, patients with allergic asthma or rhino-conjunctivitis have healthy skin, with intact innate immune mechanism, and are therefore able to better defend themselves against microorganism colonization.

We found that *S. aureus* induced lymphoproliferative responses and the secretion of TH1 and TH2 cytokines in

cultured PBMC both from AD and non-allergic persons, which is not too surprising given that both populations may have had contact with *S. aureus*. Interestingly, we did not observe relevant lymphoproliferative response or cytokine secretion when PBMC were stimulated with *E. coli* proteins, which may be explained as an effect of selective tolerance to gut bacteria. The non-specific activation of skin T-cells by *S. aureus* superantigens has been proposed as one pathomechanism by which *S. aureus* contributes to skin inflammation in AD. The presence of specific IgE-reactive antigens could represent another pathomechanism in AD because they cross-link mast cells and induce specific T-cell responses. Our data demonstrate that *S. aureus* can induce immediate and late phase skin reactions indicating that IgE and T-cell recognition of *S. aureus* allergens may contribute to skin inflammation in AD. Therefore, *S. aureus* should be considered as an allergen source for AD patients and future studies may investigate the allergenic potential of gut bacteria as well.

Our results thus provide another explanation how bacteria may contribute to the pathogenesis of AD and could open new avenues for diagnostic and therapeutic strategies. A better understanding of the individual antigens of *S. aureus* should provide component-resolved recombinant diagnostics, which may help to identify patients who benefit from antibiotic treatment and lead to the development of therapies targeting bacteria involved in the pathogenicity of AD.

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Conflict of Interest

The authors state no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Description of gut bacterial species.

Table S2. Identification of *S. aureus* and *E. coli* proteins in IgE immune complexes

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Supplementary Table 1. Description of gut bacterial species.

No.	Bacterial species	ATCC / DSM No.	Colonizing area in gut	Gram stain	Conditions	Additional information
1	<i>Streptococcus salivarius</i> subsp. <i>salivarius</i>	DSM 20560	Ileum	Positive	Aerobe	Andrewes and Horder 1906
2	<i>Enterococcus faecalis</i>	ATCC 29212	Ileum	Positive	Aerobe	(Andrewes and Horder) Schleifer and Kilpper-Balz
3	<i>Klebsiella pneumoniae</i>	DSM 16609	Ileum	Negative	Aerobe	(Schroeter 1886) Trevisan 1887
4	<i>Bacteroides fragilis</i>	DSM 2151	Ileum	Negative	Obligate anaerobe	(Veillon and Zuber 1898) Castellani and Chalmers 1919
5	<i>Bifidobacterium adolescentis</i>	DSM 20083	Colon	Positive	Anaerobe	Reuter 1963
6	<i>Lactobacillus acidophilus</i>	DSM 20079	Colon	Positive	Facultative anaerobe	(Moro 1900) Hansen and Mocquot 1970
7	<i>Clostridium ramosum</i>	DSM 1402	Colon	Positive	Anaerobe	(Veillon and Zuber 1898) Holderman et al. 1971

Supplementary Table 2. Identification of *S. aureus* and *E. coli* proteins in IgE immune complexes**(A) *S. aureus* proteins**

Patient	Reactive protein	Species	Accession number	MW of protein (Da)	Score	Database
A	DNA-binding protein HU	<i>S. aureus</i>	DBH_STAAC	9620	254	Swiss-Prot
	Flagellin	<i>Shigella flexneri</i>	FLIC_SHIFL	56603	76	Swiss-Prot
	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	<i>S. aureus</i>	ODP2_STAAC	46382	56	Swiss-Prot
	DNA-binding protein HU-1	<i>Bacillus halodurans</i>	DBH1_BACHD	9657	56	Swiss-Prot
	Ribosomal RNA small subunit methyltransferase	<i>Mycobacterium marinum</i>	RSMH_MYCMM	37524	55	Swiss-Prot
	Regulator of ribonuclease activity A	<i>Pseudomonas syringae</i>	RRAA_PSE14	17861	51	Swiss-Prot

(B) *E. coli* proteins

Patient	Reactive protein	Species	Accession number	MW of protein (Da)	Score	Database
A	Flagellin	<i>Shigella flexneri</i>	FLIC_SHIFL	56603	675	Swiss-Prot
	50S ribosomal protein L17	<i>E. coli</i>	RL17_ECO24	14414	225	Swiss-Prot
	Glyceroldehyde-3-phosphatase dehydrogenase A	<i>E. coli</i>	G3P1_ECO57	35684	147	Swiss-Prot
	Flagellin	<i>Salmonella berta</i>	FLIC_SALBE	53245	141	Swiss-Prot
	30S ribosomal protein S4	<i>E. coli</i>	RS4_ECO24	23513	115	Swiss-Prot
	Flagellin	<i>Bordetella bronchiseptica</i>	FLAA_BORBR	40508	84	Swiss-Prot
	30S ribosomal protein S4	<i>Aeromonas hydrophila</i>	RS4_AERHH	23507	77	Swiss-Prot
	30S ribosomal protein S4	<i>Alkaliphilus metalliredigens</i>	RS4_ALKMQ	23616	63	Swiss-Prot
	50S ribosomal protein L17	<i>Anaeromyxobacter dehalogenans</i>	RL17_ANAD2	18468	58	Swiss-Prot
	Dihydroxy-acid dehydratase	<i>Corynebacterium kroppenstedtii</i>	ILVD_CORK4	65348	55	Swiss-Prot
	30S ribosomal protein S5	<i>E. coli</i>	RS5_ECO24	17592	55	Swiss-Prot
	Chromosomal replication initiator protein dnaA	<i>Helicobacter pylori</i>	DNAA_HELPH	51526	52	Swiss-Prot
	Dihydroxy-acid dehydratase	<i>Polaromonas naphthalenivorans</i>	ILVD_POLNA	59238	51	Swiss-Prot
B	Flagellin	<i>Shigella flexneri</i>	FLIC_SHIFL	56603	403	Swiss-Prot
	Flagellin	<i>Salmonella berta</i>	FLIC_SALBE	53245	234	Swiss-Prot
	30S ribosomal protein S4	<i>Aeromonas hydrophila</i>	RS4_AERHH	23507	83	Swiss-Prot
	30S ribosomal protein S4	<i>Alkaliphilus metalliredigens</i>	RS4_ALKMQ	23616	67	Swiss-Prot
	Bifunctional protein pyrR	<i>Corynebacterium efficiens</i>	PYRR_COREF	21002	53	Swiss-Prot
	Chromosomal replication initiator protein dnaA	<i>Mannheimia succiniciproducens</i>	DNAA_MANSM	51806	53	Swiss-Prot
	50S ribosomal protein L16	<i>E. coli</i>	RL16_ECO24	15271	52	Swiss-Prot
C	Flagellin	<i>Shigella flexneri</i>	FLIC_SHIFL	56603	400	Swiss-Prot
	Flagellin	<i>Salmonella berta</i>	FLIC_SALBE	53245	148	Swiss-Prot
	50S ribosomal protein L2	<i>E. coli</i>	RL2_ECO24	29958	87	Swiss-Prot
	50S ribosomal protein L17	<i>E. coli</i>	RL17_ECO24	144414	78	Swiss-Prot
	Elongation factor G	<i>Lactobacillus delbrueckii</i>	EFG_LACDA	76564	63	Swiss-Prot
	Ribose-5-phosphate isomerase A	<i>Photobacterium luminescens</i>	RPIA_PHOLL	23224	53	Swiss-Prot
	Dihydroxy-acid dehydratase	<i>Cytophaga hutshinsonii</i>	ILVD_CYTH3	60159	51	Swiss-Prot
D	Flagellin	<i>Shigella flexneri</i>	FLIC_SHIFL	56603	256	Swiss-Prot
	Flagellin	<i>Salmonella berta</i>	FLIC_SALBE	53245	87	Swiss-Prot
	50S ribosomal protein L16	<i>E. coli</i>	RL16_ECO24	15271	57	Swiss-Prot