

***Staphylococcus aureus* fibronectin-binding protein specifically binds IgE from patients with atopic dermatitis and requires antigen presentation for cellular immune responses**

Kavita Reginald, PhD,^a Kerstin Westritschnig, MD,^a Birgit Linhart, PhD,^a Margarete Focke-Tejkl, PhD,^b Beatrice Jahn-Schmid, PhD,^a Julia Eckl-Dorna, MD, PhD,^c Annice Heratizadeh, MD,^f Angelika Stöcklinger, PhD,^g Nadja Balic,^d Susanne Spitzauer, MD,^d Verena Niederberger, MD,^c Thomas Werfel, MD,^f Josef Thalhamer, PhD,^g Stephan Weidinger, MD,ⁱ Natalija Novak, MD,^j Markus Ollert, MD,^{h,i} Alexander M. Hirschl, MD,^e and Rudolf Valenta, MD^{a,b} Vienna and Salzburg, Austria, and Hannover, Munich, and Bonn, Germany

Background: *Staphylococcus aureus* superinfections occur in more than 90% of patients with atopic dermatitis (AD) and aggravate skin inflammation. *S aureus* toxins lead to tissue damage and augment T-cell-mediated skin inflammation by a superantigen effect.

Objective: To characterize IgE-reactive proteins from *S aureus*. **Methods:** A genomic *S aureus* library was screened with IgE from patients with AD for DNA clones coding for IgE-reactive antigens. One was identified as fibronectin-binding protein (FBP). Recombinant FBP was expressed in *Escherichia coli*, purified, and tested for specific IgE reactivity in patients with AD. Its allergenic activity was studied in basophil activation experiments and T-cell cultures. The *in vivo* allergenic activity was investigated by sensitizing mice.

Results: Using IgE from patients with AD for screening of a genomic *S aureus* library, an IgE-reactive DNA clone was isolated that coded for FBP. Recombinant FBP was expressed in *E coli* and purified. It reacted specifically with IgE from patients with AD and exhibited allergenic activity in basophil degranulation assays. FBP showed specific T-cell reactivity requiring antigen presentation and induced the secretion of proinflammatory cytokines from PBMCs. Mice sensitized with FBP mounted FBP-specific IgE responses, showed FBP-specific basophil degranulation as well as FBP-specific T-cell proliferation, and mixed T_H2/T_H1 cytokine secretion.

Conclusion: Evidence is provided that specific humoral and cellular immune responses to *S aureus* antigens dependent on antigen presentation represent a novel mechanism for *S aureus*-induced skin inflammation in AD. Furthermore, FBP may be used for the development of novel diagnostic and therapeutic strategies for *S aureus* infections. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: *Staphylococcus aureus*, atopic dermatitis, bacterial antigen, bacterial allergen, allergy, IgE, superinfection, superantigen

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is usually associated with IgE-mediated allergic sensitization to a variety of exogenous allergens and affects 15% to 30% of children.^{1,2} Patients with AD often suffer not only from skin inflammation but also from severe forms of food and respiratory allergy, including asthma.³ In patients with the extrinsic type of AD, skin inflammation is mediated by exogenous allergen contact, which is based on IgE as well as T-cell recognition of these allergens.⁴ Patients with severe AD also mount IgE and T-cell reactivity against human proteins, which may induce a strong T_H1 immune response leading to keratinocyte apoptosis and eczema.⁵⁻⁸ In the case of the intrinsic type of AD, disease-causing allergens often cannot be identified.⁹

From ^athe Division of Immunopathology and ^bthe Christian Doppler Laboratory for Allergy Research, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, ^cthe Department of Otorhinolaryngology, ^dthe Institute of Medical and Chemical Laboratory Diagnostics, and ^ethe Department of Clinical Microbiology, Medical University of Vienna; ^fthe Department of Dermatology and Allergy, Division of Immunodermatology and Allergy Research, Hannover Medical School; ^gthe Department of Molecular Biology, Salzburg University; ^hthe Clinical Research Division of Molecular and Clinical Allergotoxicology, ⁱthe Department of Dermatology and Allergy, Technische Universität München, Munich; and ^jthe Department of Dermatology and Allergy, University of Bonn.

Supported by grants F1804, F1815, and F1818 from the Austrian Science Fund; the Christian Doppler Research Association, Vienna, Austria; and a research grant from Biomay, Vienna, Austria. B.J.-S. is supported by grant P20011-B13 from the Austrian Science Fund.

Disclosure of potential conflict of interest: V. Niederberger receives research support from the Austrian Science Fund (FWF). J. Thalhamer has consultant arrangements with Biomay AG and receives research support from Biomay AG, Christian Doppler

Forschungsgesellschaft, and the Austrian Science Foundation. S. Weidinger receives research support from the German Research Council and the Bundesministerium für Bildung und Forschung. N. Novak receives research support from the German Research Council and is a lecturer for Phadia. R. Valenta receives research support from the Austrian Science Fund, the Christian Doppler Research Association, Biomay, and Phadia and has provided legal consultation or expert witness testimony in cases related to allergy therapy and allergy diagnosis. The rest of the authors have declared that they have no conflict of interest.

Received for publication May 19, 2010; revised February 15, 2011; accepted for publication February 23, 2011.

Reprint requests: Rudolf Valenta, MD, Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria. E-mail: rudolf.valenta@meduniwien.ac.at.

0091-6749/\$36.00

© 2011 American Academy of Allergy, Asthma & Immunology
doi:10.1016/j.jaci.2011.02.034

Abbreviations used

AD: Atopic dermatitis
 APC: Antigen-presenting cell
 Con A: Concanavalin A
 FBP: Fibronectin-binding protein
 FbpA: Fibronectin-binding protein A domain
 r: Recombinant
 RBL: Rat basophil leukocyte

Besides sensitization to allergens, several other mechanisms contribute to AD. Patients with AD often have impaired skin barrier function, which is associated with mutations in the filaggrin gene.¹⁰⁻¹² In addition, individuals with AD may display a significant decrease in the expression of antimicrobial peptides and exhibit innate immune defects, which explains the high susceptibility to secondary skin infections with bacteria, fungi, and viruses.¹³

In fact, more than 90% of patients with AD have skin superinfection with *Staphylococcus aureus* and experience heavy exacerbations of skin inflammation as a result of *S aureus* infection.¹⁴ *S aureus* produces several enterotoxins that stimulate T cells in a polyclonal fashion without requiring antigen presentation. This activity is restricted to the V β chain and hence has been designated the superantigen effect.^{15,16} The superantigen effect leads to an augmentation of T-cell-mediated skin inflammation and also stimulates macrophages, keratinocytes, and MHC-expressing cells.²

Several research groups provided evidence that *S aureus* may contain IgE-reactive antigens. However, so far, only *S aureus* superantigens have been identified as IgE-reactive components in AD.^{17,18}

In this study, we report for the first time the molecular and immunological characterization of an IgE-reactive protein from *S aureus* that requires antigen presentation to induce T-cell responses.

METHODS**Characterization of patients with AD**

Ninety-five patients with AD fulfilling the criteria of Hanifin and Rajka¹⁹ were enrolled in this study. A summary of demographics for AD, allergic rhinoconjunctivitis, patients with asthma without AD, and controls without allergy is shown in Table I, and a more complete clinical description of these patients is shown in this article's Tables E1 and E2 in the Online Repository at www.jacionline.org. Patients 40 and 43 have already been described.⁷ Bacterial infections in patients with AD were identified by doctor-diagnosed clinically visible bacterial infection. The patients and control persons gave their written consent. The study was approved by the ethics committee of the Hannover Medical School, and anonymized sera were analyzed in a blinded manner in Vienna with the permission of the local ethics committee.

The presence of *S aureus*-specific IgE in AD sera used for screening of the *S aureus* library was determined by IgE immunoblotting to *S aureus* (subspecies Rosenbach, ATCC 25923) extracts. The protein extract was prepared from overnight cultures of *S aureus*, harvested by centrifugation, washed with PBS, and homogenized by using Ultra Turrax (IKA Labortechnik, Staufen, Germany) and glass beads. Preparative SDS-PAGE²⁰ separated bacterial extract was blotted onto nitrocellulose²¹ (Schleicher & Schuell, Dassel, Germany). *S aureus*-specific IgE was detected by IgE immunoblotting as described.²²

IgE immunoscreening of a genomic *S aureus* library with sera from patients with AD

See this article's [Methods](#) in the Online Repository at www.jacionline.org.

TABLE I. Summary of demographics of patients with AD, patients with rhinoconjunctivitis and asthma without AD, and individuals without allergy

Variable	AD	AR	AS	NA
Patients, n	95	21	17	17
Mean age (y) (range)	34.4 (1-72)	34.1 (22-63)	32.7 (20-67)	36.2 (25-51)
Male/female sex, n	45/50	7/14	8/9	5/12
Symptoms				
AD, n (%)	95 (100)	0 (0)	0 (0)	0 (0)
AS, n (%)	38 (40)	0 (0)	17 (100)	0 (0)
AR, n (%)	33 (34.7)	21 (100)	15 (88)	0 (0)
RC, n (%)	17 (17.9)	21 (100)	15 (88)	0 (0)
Bacterial infection				
Yes, n (%)	15 (15.8)	0 (0)	0 (0)	0 (0)
No, n (%)	63 (66.3)	21 (100)	17 (100)	17 (100)
ND, n (%)	17 (17.9)	0 (0)	0 (0)	0 (0)
Anti-FBP IgE, n (%)	34 (36)	0 (0)	0 (0)	0 (0)

AR, Allergic rhinitis; AS, asthma; NA, nonallergic; ND, not determined; RC, rhinoconjunctivitis.

Expression and purification of recombinant *S aureus* fibronectin-binding protein in *Escherichia coli*

See this article's [Methods](#) in the Online Repository.

IgE and IgG subclass reactivity of recombinant fibronectin-binding protein, IgE epitope mapping, and basophil degranulation experiments

Nitrocellulose-blotted recombinant (r) fibronectin-binding protein (FBP) and rFBP fragments were exposed to sera from patients with AD, patients with asthma or rhinitis without allergic skin manifestations, or persons without allergy, or buffer alone. Bound IgE was detected as described.²² The levels of FBP-specific IgG₁, IgG₂, and IgG₄ antibodies in sera from patients with AD (n = 78) and individuals without allergy (n = 15) were measured in duplicate by ELISA.²³

To analyze the allergenic activity of FBP, rat basophil leukocyte (RBL) cells expressing the human high-affinity IgE receptor, Fc ϵ RI (1 $\times$ 10⁵ cells/well), were loaded with serum IgE from patients with AD or individuals without allergy (1:30 serum dilution) and exposed to increasing concentrations of FBP. β -Hexosaminidase release was measured in triplicate as described.²⁴

FBP-specific lymphoproliferation and cytokine responses

See this article's [Methods](#) in the Online Repository.

Assay for measuring superantigen activity

rFBP and known *S aureus* superantigens (*Staphylococcus enterotoxin* A, B, E; Toxin Technology Inc, Sarasota, Fla) were tested for their ability to induce proliferation in T-cell lines that were originally selected for reactivity to the major mugwort allergen Art v 1²⁵ from 5 persons with allergy. Cells were cultured in 96-well plates (Nunc, Roskilde, Denmark) at 3 $\times$ 10⁴ cells/well and stimulated with different concentrations of FBP (200 ng, 1 μ g, 5 μ g per well), SEA, SEB, SEE (10 ng, 50 ng, 250 ng per well), or medium alone at 37°C with 5% CO₂.^{26,27} After 48 hours, ³H-thymidine was added, and the amount of incorporation was determined by scintillation counting 16 hours later. Results are shown for 5 μ g/well rFBP and 250 ng/well superantigens as means of duplicate experiments.

FBP-specific T-cell proliferation using viable or fixed antigen-presenting cells

See this article's [Methods](#) in the Online Repository.

ATGGCTTATGATGGCTTATTACAAAGAAAATGGTTGAGTCTCTACAATTTTAAACAACAGGACGTGTTACAAAAATCAATCAACCTGATAATGACACGATACTAATGGTTGTACGTCAA
M A Y D G L F T K K M V E S L Q F L T T G R V H K I N Q P D N D T I L M V V R Q

AATAGACAAAACCATCAATTGTTATTGTCAATCCATCCAACTTTTCAAGATTACAATTGACTACTAAAAATATGATAATCCATTATCCACCCATGTTTGC GCGTGT TTTTAGAAAA
N R Q N H Q L L L S I H P N F S R L Q L T T K K Y D N P F N P P M F A R V F R K

CAGTTAGAAGGTGGTATTATCGAATCGATTAAAGCAAATGGTAAATGATCGTCGATTGAAATCGATATAAAGAGTAAAGATGAAATGGCGGATACTATTACCGCACTGTCATCCTTGAG
H L E G G I I E S I K Q I G N D R R I E I D I K S K D E I G D T I Y R T V I L E

ATTATGGGTAAACATAGTAACCTTAATTTTAGTAGATGAAAATCGCAAAATAATTGAAGGATTAAACACTTAACACCAAAATACGAATCACTATCGTACAGTAATGCCAGGATTAAATTAT
I M G K H S N L I L V D E N R K I I E G F K H L T P N T N H Y R T V M P G F N Y

GAAGCACCACTACTCAGCACAAAATAAATCTGTATGATATTACAGGTGCAGAGGTGTTGAAATATATCGATTTTAAACGAGGTAATATTGCTAAACAATTATTGAATCAGTTGAAGGA
E A P P T Q H K I N L Y D I T G A E V L K Y I D F N A G N I A K Q L L N Q F E G

TTTAGCCCTTAAATTACGAATGAAATCGTTAGTCGTCGCAATTTATGACTTCATCAACATTACCAGAAGCATTGACGAAGTAATGGCAGAAACCAAGTTACCACCTACTCTATTTT
F S P L I T N E I V S R R Q F M T S S T L P E A F D E V M A E T K L P P T P I F

CATAAAATCATGAAACAGGTAAGAGGATTTCTATTTTATAAAGTTAAATCAATTTAATGATGATACAGTTACATACGATTCATTAAATGATTTGCTTGATCGTTTATGATGCGCGT
H K N H E T G K E D F Y F I K L N Q F N D D T V T Y D S L N D L L D R F Y D A R

GGCGAAGTGAACGCGTTAAACAACGTGCGAATGATTTAGTTCGATTTGTTCAACAGCAGTTGCACAAATATCAAAATAAATAGCGAAGTTGATTGAAGAATATGAGCAGTCTAAAAAT
G E R E R V K Q R A N D L V R F V Q Q Q L H K Y Q N K L A K L I E E Y E Q S K N

AAAGATACTGAACAGTTATATGGTGAATTGATCACTGCTAATATATATCGAATTAAGCAAGGCGATAAAGAAGTGACGCGATTGAATTATTATACGAATGAAGAAGTTGTCATTCCTTTA
K D T E Q L Y G E L I T A N I Y R I K Q G D K E V T A L N Y Y T N E E V V I P L

AATCCTACAAAATCCCATCAGCAAAATGCTCAATATTATTATAAACAATATAATCGTATGAAAACAGAGAAACGTGAATTACAAATCAAAATTCGAATGACGAAAGACAAATATAGATTAT
N P T K S P S A N A Q Y Y Y K Q Y N R M K T R E R E L Q H Q I Q L T K D N I D Y

TTTTCAACAATCGAACAACAATTACATCATATTTCTGTCCATGACATTGATGAAATAGAGATGAATTAGCAGAACAAAGCTTTATGAAACAGCGTAAAAATCAAACTAAGAAAAAGAAA
F S T I E Q Q L H H I S V H D I D E I R D E L A E Q G F M K Q R K N Q T K K K K

GCGCAGATTCAATTACAACATTATGTATCAACTGATGGCGACGATATATATGTTGGTAAGAATAACAAGCAAAATGATTATTTAACAATAAAAAAGCTAAAAAACTCACACATGGTTA
A Q I Q L Q H Y V S T D G D D I Y V G K N N K Q N D Y L T N K K A K K T H T W L

CACACAAAAGATATTCCTGGTTCACATGTCGTTATATTTAATGATGCACCAAGTGATACGACAATCAAGGAAGCGGCTATGTTAGCAGGATACCTTTTCAAAAGCTGGTAATCTGGACAA
H T K D I P G S H V V I F N D A P S D T T I K E A A M L A G Y F S K A G N S G Q

ATACCTGTTGATTATACATTAAATTAATAAATGTGCATAAACCATCAGGTGCAAGCGCTGGGTTTGTAACATATGACAATCAAAAACTTTGTATGCTACACCTGATTATGAACCTATTCAA
I P V D Y T L I K N V H K P S G A K P G F V T Y D N Q K T L Y A T P D Y E L I Q

AAAATGAAACAATCATAA
K M K Q S *

FIG 1. Sequence of *S aureus* FBP. The nucleotides and deduced amino acids of FBP (GenBank ID HM245235) are shown. *TAA stop codon. The *S aureus* FBP protein contains 2 domains, FbpA (yellow) and domain DUF814 (green). IgE-reactive fragments determined by mass spectrometry are underlined (fragment 1, solid line; fragment 2, broken line).

Allergic sensitization of mice with rFBP

See this article's [Methods](#) in the Online Repository.

Statistical analysis of data

Correlation between different parameters was tested by the Wilcoxon-Mann-Whitney *U* tests using SPSS software (SPSS Inc, Chicago, Ill) and adjusted by using the Bonferroni-Holm method. Results were considered statistically significant when $P < .05$.

RESULTS

IgE-based isolation of a DNA encoding an *S aureus* FBP from a genomic *S aureus* library

A λ gt11 genomic library of *S aureus* was screened with serum IgE from 6 patients with AD with IgE reactivity to nitrocellulose-blotted *S aureus* extracts. In total, 11 IgE-reactive phage clones were isolated, and their DNAs were sequenced. According to sequence comparisons, we found that each of these clones contained DNAs coding for a FBP.

The FBP cDNA consisted of 1698 base pairs (GenBank ID HM245235), which translate into a 565-amino acid protein having a theoretic molecular weight of 65.8 kD. A search on

the Pfam database (<http://pfam.sanger.ac.uk/>) revealed that FBP contains 2 domains, a FBP A domain (FbpA) spanning amino acids 4 to 442 and DUF814 (domain of unknown function) corresponding to amino acids 447 to 533 (Fig 1). There were 8 entries in GenBank of amino acid sequences from *S aureus* that shared more than 98% sequence identity with FBP and therefore appear to be isoforms of the same protein (see this article's [Table E3](#) in the Online Repository at www.jacionline.org). These isoforms had been identified as the result of a genome-wide search for virulence and resistance proteins in different *S aureus* strains.²⁸⁻³⁴ We then investigated whether other pathogenic bacteria also express proteins that are similar to FBP. [Table E4](#) shows that the sequence identities of FBP to homologous proteins from several microbes that are rather low, ranging from 42.7% (*Listeria monocytogenes*) to 24.4% (*Borrelia burgdorferi*).

Expression and purification of *S aureus* FBP in *E coli*

Recombinant *S aureus* FBP was expressed as a hexahistidine-tagged protein in *E coli*. The complete recombinant protein migrated in SDS-PAGE at a molecular weight of approximately 65 kD and reacted specifically with anti-hexahistidine antibodies. Interestingly, the protein reproducibly liberated defined degradation

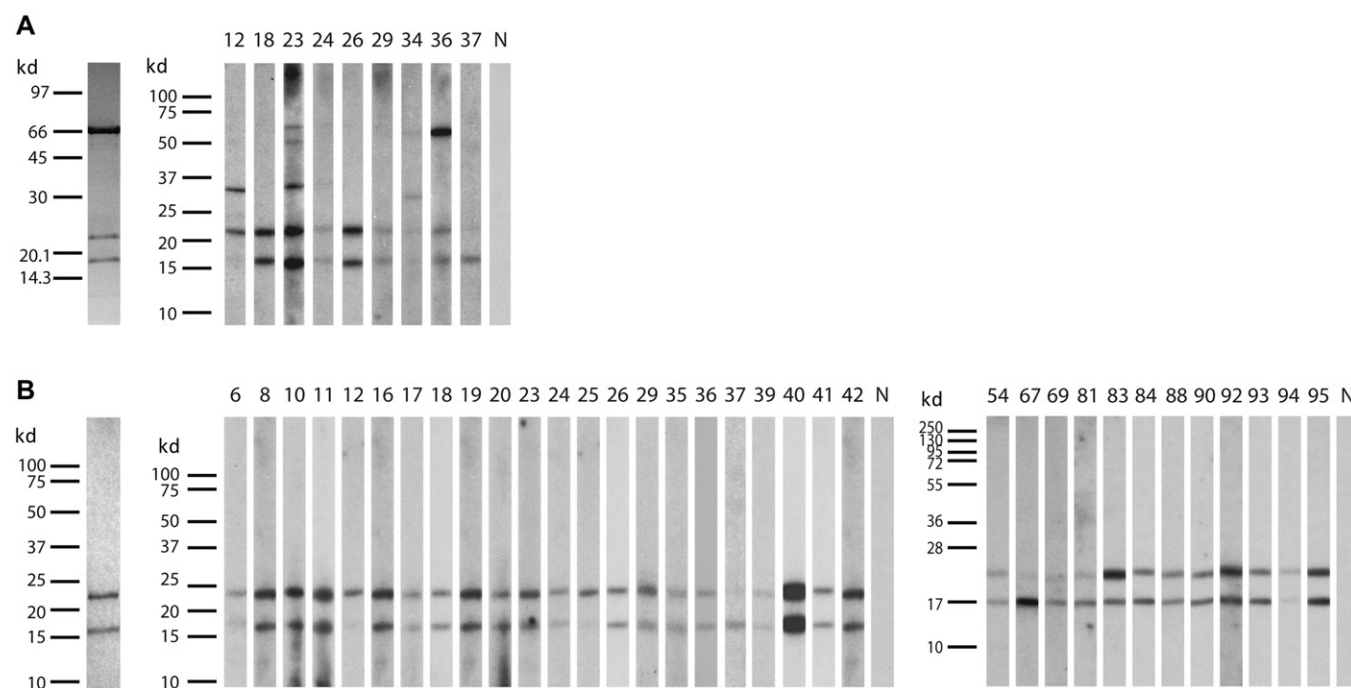


FIG 2. Purification, IgE reactivity, and epitope mapping of recombinant FBP. **A**, *Left panel* shows an SDS-PAGE containing intact affinity-purified rFBP and rFBP fragments (fragments 1 and 2). IgE reactivity of the nitrocellulose-blotted rFBP preparations (*right panel*, sera from patients with AD, lanes 12-37; sera from a person without allergy, lane N). **B**, SDS-PAGE of purified rFBP fragments (*left*) and IgE reactivity (*right*, sera from patients with AD, lanes 6-95; sera from a person without allergy, lane N).

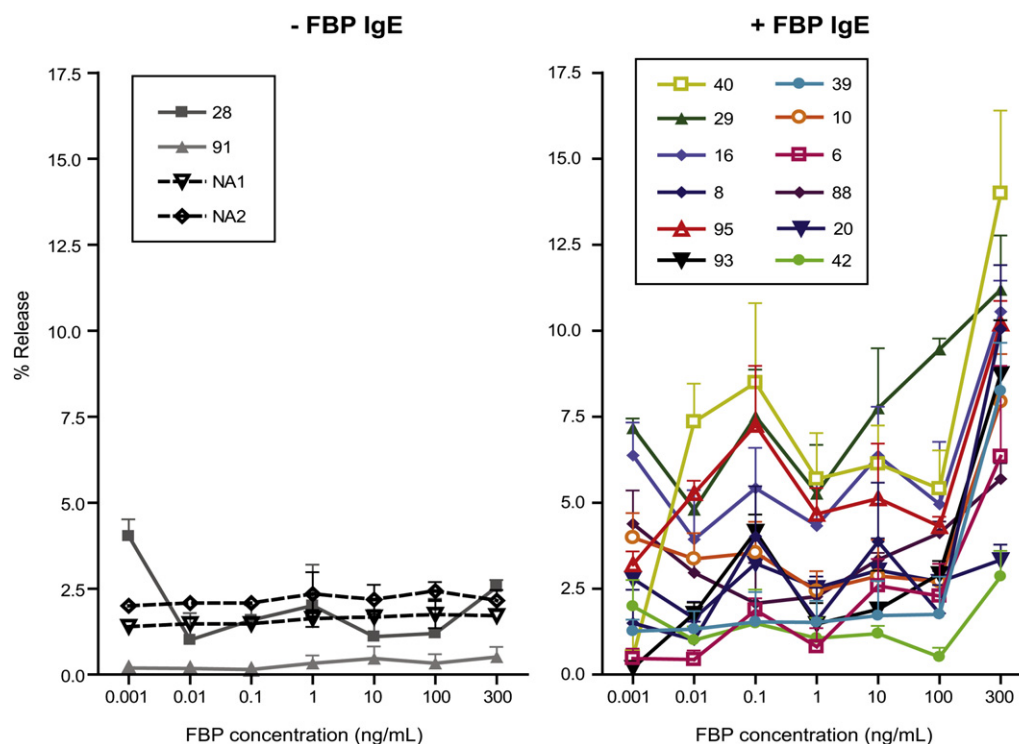


FIG 3. Allergenic activity of rFBP. RBL cells expressing human FcεRI were loaded with serum IgE from patients with AD with FBP-specific IgE (*right panel*), or patients with AD and nonallergic (NA) individuals without FBP-specific IgE (*left panel*) and challenged with rFBP. Percentages of total β-hexosaminidase releases are shown.

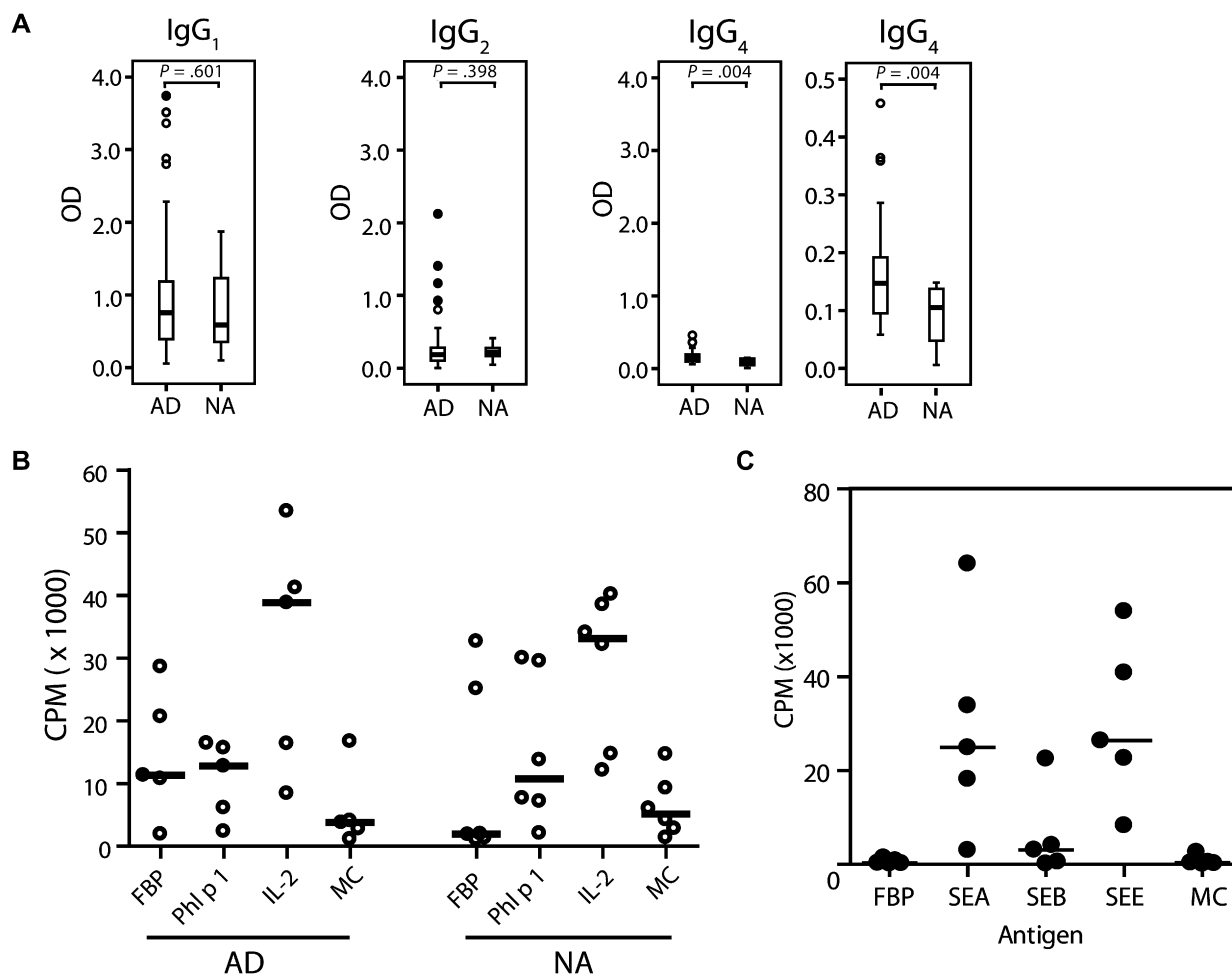


FIG 4. Immune reactivity of rFBP. **A**, FBP-specific levels of IgG₁, IgG₂ and IgG₄ (2 different scales for the y-axis) antibodies in patients with AD and nonallergic (NA) individuals. Open circles represent outliers, and filled circles represent extreme values. **B**, PBMCs from patients with AD or NA individuals were stimulated with FBP, Phl p 1, IL-2, or medium control (MC), and mean lymphoproliferative responses of triplicate measurements are shown. Horizontal lines denote the median value of each group. **C**, T-cell lines from 5 individuals with allergy were stimulated in duplicates with FBP, SEA, SEB, SEE, or MC, and the lymphoproliferative responses are shown as counts per minute (CPM).

products during expression and purification, of which the 2 predominant fragments (fragments 1 and 2) migrated at 22 and 17 kD in SDS-PAGE (Fig 2). The nature of these fragments was investigated by probing *E coli* expressing the FBP 2 hours postinduction with anti-hexahistidine antibodies. We found that both fragments reacted with the anti-hexahistidine antibodies and hence represent C-terminal fragments of FBP. Next we performed a sequence analysis of the recombinant fragments by using tryptic digestion of the gel-excised fragments and mass spectrometry. The latter studies unambiguously showed that the fragments represent mainly the DUF814 domain of FBP (Figs 1 and 2). The degradation process thus seems to separate the FbpA from the DUF814 domain.

***S aureus* FBP is an IgE-reactive protein with allergenic activity containing major IgE epitopes in the DUF814 domain**

The IgE reactivity of *S aureus* FBP was analyzed by using sera from 95 patients with AD and 17 persons without allergy. Table I shows the summary of demographics of the patients with AD,

patients with respiratory allergy without AD, and individuals without allergy.

In a first set of experiments, sera were tested for IgE reactivity with recombinant preparations containing intact *S aureus* FBP and fragments thereof (Fig 2, A). As exemplified in Fig 2, A, the majority of patients with specific IgE reactivity recognized the 17-kD and 22-kD fragments, whereas the full-length protein showed weak IgE reactivity. We therefore also tested sera with recombinant *S aureus* FBP preparations containing mainly the 17-kD and 22-kD bands and, as exemplified in Fig 2, B, found that 36% of the patients with AD tested showed specific IgE reactivity to these fragments. Sera from 17 individuals with asthma and from 21 individuals with rhinoconjunctivitis but without allergic skin manifestations showed no IgE reactivity to FBP (data not shown).

Patients with AD with high total IgE levels (>1000 kU/L) showed IgE binding to recombinant FBP more frequently than patients with low total IgE (<200 kU/L). However, there were also patients with low total IgE levels showing anti-FBP IgE antibodies (eg, patients 10, 11, and 18), demonstrating that the

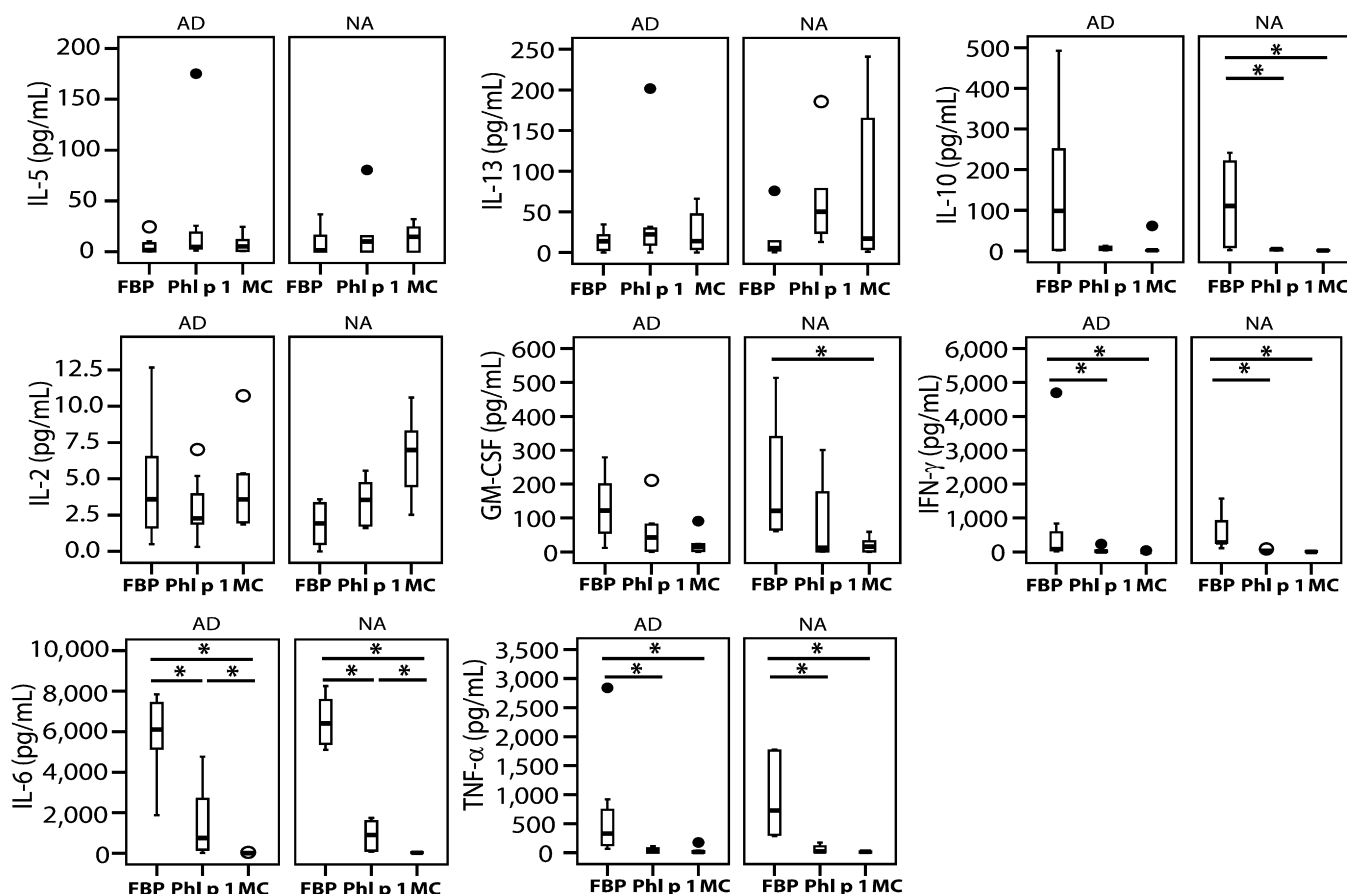


FIG 5. Cytokine releases induced from PBMCs of patients with AD and nonallergic (NA) individuals by FBP. PBMCs were stimulated with FBP, Phl p 1, or medium control (MC), and the amounts of released cytokines are presented. Statistically significant differences between groups are marked. * $P < .05$.

IgE-binding observed was specific. Some patients (eg, patients 61, 78, and 83) with high total IgE levels showed no relevant FBP-specific IgE. Hence, although elevated total serum IgE levels were often associated with IgE reactivity to recombinant FBP, it was not a prerequisite for IgE binding (Fig 2, B; Table E1).

Next we investigated whether the presence of FBP-specific IgE was related to the severity of AD. According to the available Scoring Atopic Dermatitis data, 62 out of the 95 patients (ie, 65%) could be classified as mild (0-24), moderate (25-50), or severe (>50). We found that 6%, 25%, and 36% of these patients had FBP-specific IgE reactivity in the mild, moderate, and severe groups, respectively (Fig 2; Table E1). Hence, FBP-specific IgE reactivity seems to be more frequent in severe AD.

From the 95 AD patients tested, 15 had bacterial skin infections, and 63 did not have bacterial infections. For 17 patients, information about bacterial infection was not available. Patients with *S aureus* skin superinfections showed a higher percentage of IgE reactivity to FBP (6 of 15; 40%), than those without skin superinfections (18 of 63; 29%; Fig 2; Table E1).

Next, the allergenic activity of *S aureus* FBP was studied. RBL cells expressing the human FcεRI receptor were loaded with serum IgE from patients with AD where sufficient serum was available and individuals without allergy. Fig 3 shows that most of the sera containing FBP-specific IgE gave degranulation, whereas no relevant degranulation was observed for sera without FBP-specific IgE at the tested FBP concentrations.

***S aureus* FBP-specific IgG subclass response in patients with AD is indicative of a T_H2 response**

The median *S aureus* FBP-specific IgG₄ levels were significantly higher in patients with AD than nonatopic persons, indicating a T_H2 bias (Fig 4, A; $P = .004$). No significant differences were found regarding the *S aureus* FBP-specific IgG₁ and IgG₂ levels when patients with AD were compared with nonallergic persons. Levels of FBP-specific IgG₃ were not measured because we were limited by the amount of sera and because it is scarcely found in atopic persons.²³

***S aureus* FBP requires antigen presentation to induce specific lymphoproliferative responses and does not act as superantigen**

We then measured the lymphoproliferative responses of PBMCs isolated from 5 patients with AD (Table E1: patients 39, 40, 41, 42, and 92) and 6 individuals without allergy to *S aureus* FBP, major timothy grass allergen, Phl p 1, or medium alone (Fig 4, B). There was no statistically significant difference between FBP and Phl p 1-specific lymphoproliferative responses in patients with AD and persons without allergy.

All so far characterized IgE-reactive *S aureus* components are superantigens. We therefore exposed T-cell lines from 5 allergic individuals with FBP or known *S aureus* superantigens such as SEA, SEB, and SEE in the absence of antigen-presenting cells

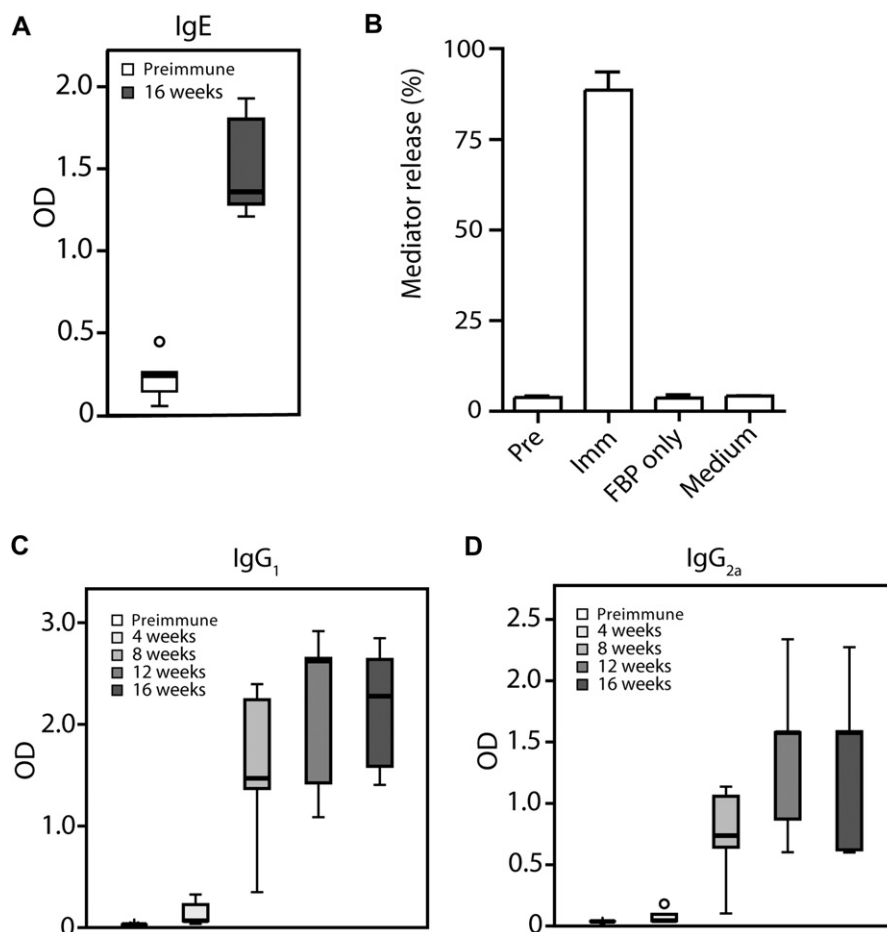


FIG 6. FBP induces an allergic immune response in Balb/c mice. **A**, FBP-specific IgE levels were measured in a group of FBP-sensitized mice at different times. Open circles represent outliers. **B**, β -Hexosaminidase release from RBL cells that had been sensitized with preimmune (Pre) or immune (Imm) sera and were stimulated with FBP. RBL cells without sera exposure were stimulated with FBP or medium alone. Levels of FBP-specific IgG₁ (**C**) and IgG_{2a} (**D**) were measured as for IgE in **A**.

(APCs; Fig 4, C). Even when used at 20-fold higher concentration compared with the superantigens, FBP did not induce proliferative responses. Superantigens SEA and SEE induced equally high levels of proliferation in all T-cell lines tested, whereas SEB induced strong proliferation only in 1 of the 5 T-cell lines. No proliferation was observed when T cells were kept in medium alone (Fig 4, C).

In another experiment, T cells of patients with AD stimulated with rFBP did not proliferate in the absence of APCs, or when fixed APCs were used (see this article's Fig E1 in the Online Repository at www.jacionline.org). In contrast, T cells stimulated with the superantigen SEA proliferated even in the absence of functional APCs. These results show that the induction of T-cell proliferation by FBP is dependent on the antigen processing by viable APCs.

***S aureus* FBP induces the release of proinflammatory cytokines IL-6, IFN- γ , and TNF- α in cultured PBMCs**

FBP induced high levels of the proinflammatory cytokines IFN- γ , IL-6, and TNF- α in PBMCs from patients with AD and

persons without allergy compared to stimulation with grass pollen allergen Phl p 1 or medium only ($P < .05$; Fig 5). The relatively strong induction of IFN- γ , IL-6, and TNF- α by FBP most likely does not result from endotoxin contaminations because the endotoxin concentration recorded for Phl p 1, which also had been purified from *E coli*, was 3-fold higher (83.3 endotoxin units/mL) compared with FBP.

***S aureus* FBP induces an allergic immune response in mice**

Fig 6, A, shows that FBP-sensitized mice develop robust FBP-specific IgE antibody responses. When RBL cells were loaded with IgE from sensitized mice, strong degranulation could be induced by addition of FBP, whereas no degranulation was observed when cells were loaded with the preimmune sera (Fig 6, B). No degranulation was seen when cells had not been loaded with IgE and subsequently were exposed to FBP or medium alone (Fig 6, B). FBP-sensitized mice also developed high levels of specific IgG₁ antibodies and to a lower extent, FBP-specific IgG_{2a} antibodies (Fig 6, C and D).

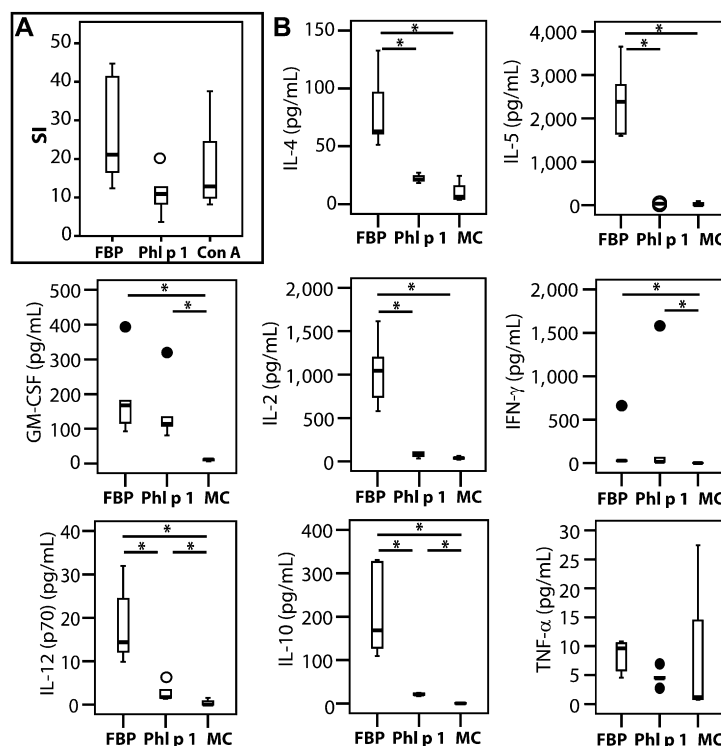


FIG 7. FBP-specific lymphoproliferation and cytokine responses. **A**, Lymphoproliferative responses toward different agonists as determined by ^3H -thymidine incorporation are shown as stimulation indices of triplicate measurements. Open circles represent outliers, whereas filled circles represent extreme values. **B**, FBP-specific cytokine responses. Statistically significant differences between groups are marked. * $P < .05$. MC, Medium control; SI, stimulation index.

FBP-sensitized mice exhibit strong lymphoproliferative and $\text{T}_\text{H}2$ cytokine responses

Splenocytes from FBP-sensitized mice were stimulated with FBP, the grass pollen allergen Phl p 1, or concanavalin A (Con A). FBP induced even higher lymphoproliferative responses than Con A, whereas Phl p 1 induced only weak proliferation (Fig 7, A). We also analyzed supernatants from FBP-stimulated splenocytes regarding the profile of cytokines and found that FBP augmented the production of the $\text{T}_\text{H}2$ and $\text{T}_\text{H}1$ cytokines (Fig 7, B).

DISCUSSION

In this study, we report the detailed molecular characterization of a novel IgE-reactive protein from *S aureus*. It is different from previously described IgE-reactive superantigens because it requires antigen presentation to induce T-cell responses. The protein was identified by sequence comparisons as a novel class of FBPs. More than one third of the patients with AD showed specific IgE reactivity to FBP. Generally, AD patients with higher total serum IgE levels reacted more often with FBP, but also patients with low total serum IgE levels showed specific IgE reactivity to FBP. AD patients with more severe symptoms and patients with *S aureus* skin superinfections also seemed to exhibit FBP-specific IgE reactivity more frequently than patients with mild AD symptoms and without bacterial skin infections. FBP-specific IgE reactivity was found in patients with AD and was not observed in patients with mainly mild forms of respiratory allergies.

rFBP also exhibited allergenic activity in basophil degranulation assays and stimulated the proliferation of T cells in an

antigen-specific manner and thus is different from previously described *S aureus* superantigens.

When recombinant FBP was produced in *E coli*, it consistently liberated 2 fragments of 22 and 17 kD (fragments 1 and 2). Interestingly, the majority of the patients with AD tested in this study showed stronger and more frequent IgE reactivity to fragments 1 and 2 of FBP than to the full-length FBP protein. This demonstrates that the predominant IgE epitopes of FBP are localized on these fragments, which overlap with the DUF814 domain of the protein.

Fibronectin-binding proteins are important in *S aureus* infections because the central mechanism of adhesion and invasion of the epithelial barrier by *S aureus* occurs via binding of FBPs to fibronectin.^{35,36} FBP identified by us represents a novel class of FBPs that is distinct in sequence (<15% sequence identity) from 2 earlier characterized *S aureus* FBPs.^{37,38}

Moreover, FBP is the first IgE-reactive protein from *S aureus* requiring antigen presentation for cellular responses because all of the previously described IgE-reactive *S aureus* antigens had superantigen activity. By contrast, FBP induced potent and specific lymphoproliferation. The stimulation of PBMCs with FBP resulted in a high release of proinflammatory cytokines, in particular of IFN- γ , IL-6, and TNF- α . In fact, a cytokine milieu that is rich in IL-6, IL-1, and TGF- β is known to promote the formation of $\text{T}_\text{H}17$ cells, which may play an important role in various inflammatory processes, including AD.³⁹ Several recent studies indeed suggest that $\text{T}_\text{H}17$ cells play a role in allergic diseases,⁴⁰ and it has been shown that epicutaneous sensitization, in particular in filaggrin-deficient mice, induces $\text{T}_\text{H}17$ responses

and skin inflammation reminiscent of AD.^{12,41} It thus remains to be investigated by *in vivo* studies whether FBP induces a T_H17 cell-mediated skin inflammation in sensitized patients with AD by clinical testing using epicutaneous application and histologic examination of punch biopsies from the challenged skin sites.

We studied the allergenic activity of FBP in a murine model by using a classic sensitization protocol based on aluminum hydroxide-adsorbed antigen for the induction of IgE-mediated allergic sensitization.⁴² Despite the fact that FBP was a bacterial protein, we found that it induced a T_H2-driven and IgE-mediated allergic immune response in mice.

FBP is recognized by a subgroup of patients with AD and hence may be used for serologic diagnosis to identify sensitized patients with AD and eventually to develop therapeutic strategies to suppress *S aureus*-induced skin inflammation in these patients.

Moreover, the identification of FBP may have implications that go beyond its role in AD. There is unmet need for the improvement of diagnosis and therapy of *S aureus* infections. Due to a low degree of sequence similarity between FBP from *S aureus* and other bacteria, it may be possible to develop a sensitive PCR-based method for the detection of *S aureus* infections. In addition, serologic assays may be developed that, on the basis of the measurement of FBP-specific antibody responses, may allow diagnosis of *S aureus* infections.

The possibilities for antibiotic treatment of *S aureus* infections are becoming more and more limited because the bacterium rapidly develops antibiotic resistance.⁴³ In fact, FBP may represent a possible candidate vaccine because it is required for the adherence of *S aureus*, and we found that it induced upon immunization high antibody titers and specific cellular responses.

In summary, we provide evidence that specific allergic immune responses to FBP from *S aureus* may represent a new pathomechanism in AD and eventually provide tools for new diagnostic and therapeutic strategies for *S aureus* infections.

Clinical implications: Specific immune responses to *S aureus* FBP may contribute to allergic inflammation in a subset of patients with AD.

REFERENCES

- Bieber T. Atopic dermatitis. *N Engl J Med* 2008;358:1483-94.
- Werfel T. The role of leukocytes, keratinocytes, and allergen-specific IgE in the development of atopic dermatitis. *J Invest Dermatol* 2009;129:1878-91.
- Spergel JM, Paller AS. Atopic dermatitis and the atopic march. *J Allergy Clin Immunol* 2003;112:S118-27.
- Darsow U, Laifauji J, Kerschenlohr K, Wollenberg A, Przybilla B, Wuthrich B, et al. The prevalence of positive reactions in the atopy patch test with aeroallergens and food allergens in subjects with atopic eczema: a European multicenter study. *Allergy* 2004;59:1318-25.
- Valenta R, Maurer D, Steiner R, Seiberler S, Sperr WR, Valent P, et al. Immunoglobulin E response to human proteins in atopic patients. *J Invest Dermatol* 1996;107:203-8.
- Natter S, Seiberler S, Hufnagl P, Binder BR, Hirschl AM, Ring J, et al. Isolation of cDNA clones coding for IgE autoantigens with serum IgE from atopic dermatitis patients. *FASEB J* 1998;12:1559-69.
- Mittermann I, Reininger R, Zimmermann M, Gangl K, Reisinger J, Aichberger KJ, et al. The IgE-reactive autoantigen Hom s 2 induces damage of respiratory epithelial cells and keratinocytes via induction of IFN-gamma. *J Invest Dermatol* 2008;128:1451-9.
- Valenta R, Mittermann I, Werfel T, Garn H, Renz H. Linking allergy to autoimmune disease. *Trends Immunol* 2009;30:109-16.
- Novak N, Bieber T. Allergic and nonallergic forms of atopic diseases. *J Allergy Clin Immunol* 2003;112:252-62.
- Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 2006;38:441-6.
- Holgate ST. The epithelium takes centre stage in asthma and atopic dermatitis. *Trends Immunol* 2007;28:248-51.
- Oyoshi MK, Murphy GF, Geha RS. Filaggrin-deficient mice exhibit TH17-dominated skin inflammation and permissiveness to epicutaneous sensitization with protein antigen. *J Allergy Clin Immunol* 2009;124:485-93, 93 e1.
- Lubbe J. Secondary infections in patients with atopic dermatitis. *Am J Clin Dermatol* 2003;4:641-54.
- Leung DY, Bieber T. Atopic dermatitis. *Lancet* 2003;361:151-60.
- Choi YW, Kotzin B, Herron L, Callahan J, Marrack P, Kappler J. Interaction of *Staphylococcus aureus* toxin "superantigens" with human T cells. *Proc Natl Acad Sci U S A* 1989;86:8941-5.
- Fraser JD, Proft T. The bacterial superantigen and superantigen-like proteins. *Immunol Rev* 2008;225:226-43.
- Hauser C, Wuthrich B, Matter L, Wilhelm JA, Schopfer K. Immune response to *Staphylococcus aureus* in atopic dermatitis. *Dermatologica* 1985;170:114-20.
- Bunikowski R, Mielke M, Skarabis H, Herz U, Bergmann RL, Wahn U, et al. Prevalence and role of serum IgE antibodies to the *Staphylococcus aureus*-derived superantigens SEA and SEB in children with atopic dermatitis. *J Allergy Clin Immunol* 1999;103:119-24.
- Hanifin J, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol (Stockh)* 1980;(suppl 92):44-7.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979;76:4350-4.
- Valenta R, Duchene M, Ebner C, Valent P, Sillaber C, Deviller P, et al. Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992;175:377-85.
- Stern DA, Riedler J, Nowak D, Braun-Fahrlander C, Swoboda I, Balic N, et al. Exposure to a farming environment has allergen-specific protective effects on TH2-dependent isotype switching in response to common inhalants. *J Allergy Clin Immunol* 2007;119:351-8.
- Schulmeister U, Hochwallner H, Swoboda I, Focke-Tejkl M, Geller B, Nystrand M, et al. Cloning, expression, and mapping of allergenic determinants of alphaS1-casein, a major cow's milk allergen. *J Immunol* 2009;182:7019-29.
- Jahn-Schmid B, Kelemen P, Himly M, Bohle B, Fischer G, Ferreira F, et al. The T cell response to Art v 1, the major mugwort pollen allergen, is dominated by one epitope. *J Immunol* 2002;169:6005-11.
- Nisini R, Matricardi PM, Fattorossi A, Biselli R, D'Amelio R. Presentation of superantigen by human T cell clones: a model of T-T cell interaction. *Eur J Immunol* 1992;22:2033-9.
- Koning F, Rust C. Staphylococcal enterotoxin-mediated human T-T cell interactions. *J Immunol* 1992;149:317-22.
- Gill SR, Fouts DE, Archer GL, Mongodin EF, Deboy RT, Ravel J, et al. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* 2005;187:2426-38.
- Baba T, Takeuchi F, Kuroda M, Yuzawa H, Aoki K, Oguchi A, et al. Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* 2002;359:1819-27.
- Herron-Olson L, Fitzgerald JR, Musser JM, Kapur V. Molecular correlates of host specialization in *Staphylococcus aureus*. *PLoS One* 2007;2:e1120.
- Ohta T, Hirakawa H, Morikawa K, Maruyama A, Inose Y, Yamashita A, et al. Nucleotide substitutions in *Staphylococcus aureus* strains, Mu50, Mu3, and N315. *DNA Res* 2004;11:51-6.
- Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, et al. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 2001;357:1225-40.
- Holden MT, Feil EJ, Lindsay JA, Peacock SJ, Day NP, Enright MC, et al. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci U S A* 2004;101:9786-91.
- Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, et al. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* 2006;367:731-9.
- Peacock SJ, Foster TJ, Cameron BJ, Berendt AR. Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells. *Microbiology* 1999;145(pt 12):3477-86.
- Sinha B, Francois PP, Nusse O, Foti M, Hartford OM, Vaudaux P, et al. Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin alpha5beta1. *Cell Microbiol* 1999;1:101-17.

37. Flock JI, Froman G, Jonsson K, Guss B, Signas C, Nilsson B, et al. Cloning and expression of the gene for a fibronectin-binding protein from *Staphylococcus aureus*. *EMBO J* 1987;6:2351-7.
38. Signas C, Raucci G, Jonsson K, Lindgren PE, Anantharamaiah GM, Hook M, et al. Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus*: use of this peptide sequence in the synthesis of biologically active peptides. *Proc Natl Acad Sci U S A* 1989;86:699-703.
39. Littman DR, Rudensky AY. Th17 and regulatory T cells in mediating and restraining inflammation. *Cell* 2010;140:845-58.
40. Romagnani S. Human Th17 cells. *Arthritis Res Ther* 2008;10:206.
41. He R, Oyoshi MK, Jin H, Geha RS. Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. *Proc Natl Acad Sci U S A* 2007;104:15817-22.
42. Hammad H, Kool M, Soullie T, Narumiya S, Trottein F, Hoogsteden HC, et al. Activation of the D prostanoid 1 receptor suppresses asthma by modulation of lung dendritic cell function and induction of regulatory T cells. *J Exp Med* 2007;204:357-67.
43. DeLeo FR, Chambers HF. Reemergence of antibiotic-resistant *Staphylococcus aureus* in the genomics era. *J Clin Invest* 2009;119:2464-74.

METHODS

IgE immunoscreening of a genomic *S aureus* library with sera from patients with AD

An *S aureus* library (strain RN 450; Stratagene, La Jolla, Calif) constructed in Lambda Zap II was used to infect *E coli* cells (XL1-Blue MRF⁺ host strain; Stratagene, East Kew, Australia) and screened with a serum pool from 6 patients with AD containing IgE antibodies against nitrocellulose-blotted *S aureus* extract as described.^{E1}

Phage DNA from IgE binding clones was isolated^{E2} and digested with *Kpn* I and *Sac* I to allow for directional cloning of the fragments in plasmid pUC18. The ligated product was transformed into *E coli* XL-1 blue. Positive clones were identified by DNA restriction analysis, and double-stranded DNA sequencing was performed (MWG, Ebersberg, Germany). The obtained cDNA and deduced amino acid sequences were compared with the sequences deposited in GenBank by using the BLAST-X algorithm.

Expression and purification of recombinant *S aureus* FBP in *E coli*

The cDNA encoding the FBP protein was PCR-amplified by using oligonucleotides (5' CAT GCC ATG GCT TAT GAT GGC TTA TTT 3'; 5' CGG AAT TCT TAA TGG TGA TGG TGA TGG TGT GAT TGT TTC ATT TTT TGA AT 3') containing a *Nco*I site (*italics*) and an *Eco*R I site (*boldface*) as well as a sequence coding for a C-terminal hexahistidine tag (*underlined*). The PCR product was cut with *Nco*I and *Eco*R I and subcloned into the corresponding sites of expression plasmid pET23d (Novagen, Madison, Wis), transformed into *E coli* BL21(DE3) (Stratagene), and grown in Luria Bertani-medium containing 100 mg/L ampicillin at 37°C. Protein expression was induced for 4 hours by the addition of 1 mM isopropyl- β -thiogalactopyranoside when the culture reached an OD₆₀₀ of 0.5. Cells were harvested by centrifugation at 4000g for 15 minutes at 4°C and lysed by using an Ultra Turrax (IKA Labortechnik, Germany) in binding buffer (100 mM NaH₂PO₄, 10 mM Tris.Cl and 8 M urea, pH 8.0). The His-tagged recombinant FBP was incubated with Ni-NTA resin (QIAGEN, Hilden, Germany) in binding buffer for 1 hour. The protein resin was then loaded on columns, washed twice with buffer B (100 mM NaH₂PO₄, 10 mM Tris.Cl, and 8 M urea, pH 6.3) followed by 2 washes with buffer C (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) followed by elution with 50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole, pH 8.0. The eluted protein was then dialyzed against distilled water, and the protein concentration was determined by the Micro-BCA Protein Assay Kit (Pierce, Rockford, Ill). Purified rFBP and fragments thereof were excised from SDS-gels and digested by using the Trypsin Profile IGD Kit (Sigma, St Louis, Mo) and then subjected to mass spectrometry on Nano-LC system coupled to a Bruker HCT Ultra ESI trap (Bruker Daltonics, Bremen, Germany). The peptides were fractionated on a C18 PepMap 100 column (3 μ m, 100 Å; Dionex LC Packings, Sunnyvale, Calif) by using a 5% to 80% acetonitrile solvent gradient. Mass spectra were recorded as described^{E3} except that generated peak lists were searched against the Swiss Prot databank by using the MASCOT (Matrix Science) search engine.

FBP-specific lymphoproliferation and cytokine responses

PBMCs were isolated from heparinized blood from patients with AD or control individuals by using Ficoll-Paque gradient centrifugation. PBMCs were resuspended in Ultraculture medium (Bio Whittaker, Walkersville, Md) supplemented with 2 mM glutamine, 50 mM β -mercaptoethanol, and 0.1 mg/mL gentamicin. Cells were cultured in sterile 96-well plates (Nunc) at 2×10^5 cells/well and stimulated with rFBP (5.0 μ g/well), rPhl p 1 (5.0 μ g/well, Bio-may), IL-2 (positive control), or medium (negative control) in triplicate at 37°C with 5% CO₂. After 6 days, 0.5 μ Ci of ³H-thymidine (Amersham, Buckinghamshire, United Kingdom) was added to each well, and cells were incubated for 16 hours. ³H-thymidine incorporation was measured by scintillation counting (Microbeta Trilux; Perkin Elmer, Waltham, Mass) and is reported as the 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-6, IL-10, IL-12, IL-13, GM-CSF, TNF- α , and IFN- γ) were measured in supernatants collected from PBMC cultures at day 6 by using xMAP Luminex fluorescent bead-based technology (Luminex Corp, Austin, Tex). The assays were performed according to the manufacturer's instructions (R&D Systems, Wiesbaden, Germany), and fluorescent signals were read on a Bio-Plex system (Bio-Rad, Hercules, Calif).

FBP-specific T-cell proliferation using viable or fixed APCs

PBMCs were isolated from heparinized whole blood of patients with allergy by Ficoll-Paque gradient. T cells were prepared from total PBMCs by negative magnetic separation (Pan T-cell isolation kit II; Miltenyi Biotec, Bergisch Gladbach, Germany). APCs were prepared from PBMCs by depletion of T cells using anti-CD3 beads (Miltenyi Biotec). Isolated T cells were tested for purity by flow cytometry. In experiments in which fixed cells were used, APCs were fixed in 2% paraformaldehyde for 10 minutes at 37°C. Fixed cells were washed twice in PBS before setup of culture. PBMCs, T cells, and T-cell APC cocultures at a ratio of 1:1 were cultured in 96-well plates (2×10^5 cells/well). Cells were left unstimulated or stimulated with rFBP (5, 1, or 0.2 μ g/well) or SEA (0.1 μ g/well). Cultures were pulsed with ³H-thymidine (GE Healthcare, Buckinghamshire, United Kingdom) at 0.5 μ Ci/well for 16 hours before harvest, and ³H-thymidine incorporation was measured using a scintillation counter (MicroBeta TriLux; PerkinElmer, Waltham, Mass). Thymidine incorporation in unstimulated cells was subtracted as background. Results are shown for 1 μ g/well rFBP and 0.1 μ g/well SEA as means of triplicate experiments.

Allergic sensitization of mice with rFBP

Female BALB/c mice 5 to 8 weeks old were purchased from Charles River Laboratories (Kisslegg, Germany) and kept under specific pathogen-free conditions. All experiments were approved by the local review board of the Medical University of Vienna and were performed in accordance with National Institutes of Health guidelines of laboratory animal care.

A group of mice (n = 5) was sensitized subcutaneously with 10 μ g rFBP, adsorbed to Al(OH)₃ (Alu-Gel-S; Serva Electrophoresis, Heidelberg, Germany) in monthly intervals.^{E4} Blood samples were taken from the tail veins, and serum was stored at -20°C until analysis.

Specific IgE, IgG₁, and IgG_{2a} antibody levels were measured in each serum sample by ELISA using 1:500 (IgG₁), 1:100 (IgG_{2a}), or 1:20 (IgE) diluted sera.

The allergenic activity of rFBP was determined by basophil degranulation experiments. RBL-2H3 cells were sensitized with 1:10-diluted mouse sera obtained before the first injection and after the fourth injection, washed, and then exposed to rFBP (0.3 μ g/mL). The release of β -hexosaminidase was determined as described.^{E4} For control purposes, RBL cells that had not been sensitized with serum IgE were exposed to rFBP (0.3 μ g/mL) or medium alone. Results were expressed as percentages of total β -hexosaminidase and represent the means of triplicate determinations.

Lymphoproliferative and cytokine responses in mice were determined in splenocyte cultures. Spleens were removed from mice 1 month after the fourth immunization. Splenocytes were isolated as described^{E4} and resuspended in complete medium (RPMI 1640, 10% FCS, 0.1 mg/mL gentamicin, and 2 mM glutamine). Single-cell suspensions were plated into 96-well round-bottom plates (Nunc) at a concentration of 2×10^5 cells/well (200 μ L) in triplicates and stimulated with Con A (0.5 μ g/well), rFBP (5.0 μ g/well), rPhl p 1 (5.0 μ g/well), or medium alone for 6 days. Splenocyte proliferation was measured as described.^{E4} Cytokines (IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IFN- γ , GM-CSF, and TNF- α) were measured after stimulation of mice splenocytes (5×10^6 cells/well) with rFBP, rPhl p 1, or Con A (15 μ g/ 50 μ L each) by using the Bio-Plex T_H1/T_H2 kit (Bio-Rad) and the Bio-Plex Manager software with standard curves derived from recombinant cytokine standards.^{E5}

REFERENCES

- E1. Valenta R, Duchene M, Pettenburger K, Sillaber C, Valent P, Bettelheim P, et al. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 1991;253:557-60.

- E2. Maniatis T, Sambrook J, Fritsch EF. Molecular cloning - a laboratory manual. Cold Spring Harbor (NY): Cold Spring Harbor Pr; 1982.
- E3. Hemmer W, Focke M, Marzban G, Swoboda I, Jarisch R, Laimer M. Identification of Bet v 1-related allergens in fig and other Moraceae fruits. *Clin Exp Allergy* 2010;40:679-87.
- E4. Linhart B, Bigenzahn S, Hartl A, Lupinek C, Thalhamer J, Valenta R, et al. Costimulation blockade inhibits allergic sensitization but does not affect established allergy in a murine model of grass pollen allergy. *J Immunol* 2007;178:3924-31.
- E5. Edlmayr J, Niespodziana K, Linhart B, Focke-Tejkl M, Westritschnig K, Scheiblhofer S, et al. A combination vaccine for allergy and rhinovirus infections based on rhinovirus-derived surface protein VP1 and a nonallergenic peptide of the major timothy grass pollen allergen Phl p 1. *J Immunol* 2009;182:6298-306.
- E6. Gill SR, Fouts DE, Archer GL, Mongodin EF, Deboy RT, Ravel J, et al. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* 2005;187:2426-38.
- E7. Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, et al. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 2001;357:1225-40.
- E8. Ohta T, Hirakawa H, Morikawa K, Maruyama A, Inose Y, Yamashita A, et al. Nucleotide substitutions in *Staphylococcus aureus* strains, Mu50, Mu3, and N315. *DNA Res* 2004;11:51-6.
- E9. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, et al. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* 2006;367:731-9.
- E10. Baba T, Takeuchi F, Kuroda M, Yuzawa H, Aoki K, Oguchi A, et al. Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* 2002;359:1819-27.
- E11. Holden MT, Feil EJ, Lindsay JA, Peacock SJ, Day NP, Enright MC, et al. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci U S A* 2004;101:9786-91.
- E12. Herron-Olson L, Fitzgerald JR, Musser JM, Kapur V. Molecular correlates of host specialization in *Staphylococcus aureus*. *PLoS One* 2007;2:e1120.

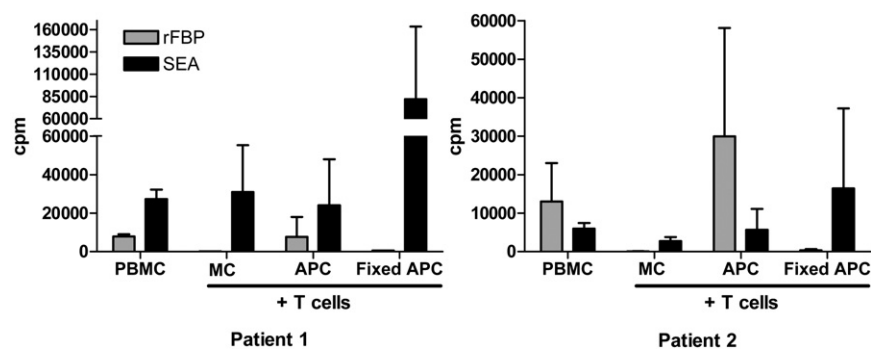


FIG E1. FBP-specific T-cell proliferation using viable or fixed APCs. Purified T cells from 2 patients with AD were cocultured with either viable or fixed APCs and stimulated with rFBP or SEA. The PBMCs of each patient served as controls. Cell proliferation responses were measured by ^3H thymidine incorporation and are displayed as counts per minute (*cpm*). *MC*, Medium control.

TABLE E1. Demographic, clinical, and serologic characterization of patients with AD

No.	Sex	Age (y)	Symptoms	SCORAD	Allergies			Total IgE (kU/L)	FBP IgE	Bacterial infection
					Pollen	Food	Other			
1	F	41	AD	ND	g, w		a, f, mi	429	—	ND
2	F	55	AD, AS, RC	ND	t, g, w	n, ap, ca	mi	1,801	—	N
3	M	1	AD	ND		n, e	mi	32.9	—	N
4	F	22	AD, RC	27	t, g			139	—	N
5	F	22	AD, AR	16	t	n, ap, ca, c		168	—	N
6	M	31	AD	ND	t, g		a, f, mi, asp	10,546	+	Y
7	F	41	AD, RC	ND			mi	1,334	—	ND
8	F	8	AD, AS	ND	t, g, w	n, mk, s	a, mi, asp	22,090	+	N
9	F	21	AD, AR	33	g	ap	f, mi	132	—	N
10	M	46	AD	ND	t	b	a, l	122	+	Y
11	F	60	AD, AS, RC	ND	t		mi	164	+	N
12	M	34	AD	ND		n, e, s, wh, so	a, f, mi, asp	23,900	+	ND
13	F	3	AD	35		n, e		281	—	N
14	M	40	AD	ND	t, g	n, e	a, mi, l	448	—	N
15	F	36	AD, RC	ND		ap	f	141	—	Y
16	F	49	AD, AS, RC	ND	t, g	n, e, wh, so	a, mi, asp, l	6,748	+	N
17	F	17	AD, AS	ND		n	a	2,222	+	ND
18	F	21	AD	19	t, w			61.8	+	N
19	M	28	AD	ND	t, g, w		a, mi, asp	39,181	+	Y
20	F	41	AD, AR	27	t, g, w	n, ap, ca, c	a, f, l	312	+	N
21	F	41	AD, AR	41	t, g, w	n, so, wh	a	158	—	N
22	M	31	AD	ND	w		wa	18.1	—	N
23	M	20	AD	ND	t, g, w	n, e, mk, s, so, ca, c	a, f, mi, asp	25,328	+	ND
24	F	21	AD, AS	ND	t, g, w		mi	9,375	+	N
25	M	23	AD, AR	ND		n, e, mk, s, wh, so	mi	9,265	+	N
26	F	31	AD, RC	ND			a	3,736	+	N
27	F	26	AD	17	ND	ND	ND	15.3	—	N
28	F	66	AD, AR, RC	25	t	n, mk, ap, ca, c	a, mi	793	—	N
29	M	65	AD	40	g		mi	3,317	+	N
30	F	18	AD	65	ND	ND	ND	73.3	—	N
31	F	23	AD	31	t	n, ap, ca	a, mi	1,122	—	N
32	M	31	AD, AS, RC	11	t, g, w	n, ap, ca, c, po, wh, so	a	412	—	N
33	M	41	AD	14	t, g		a, mi	166	—	N
34	F	61	AD, AS, RC	20	g		mi	7,290	—	N
35	M	26	AD, AS	26	t, g, w	n, e, mk, s, b, wh, so, ap, ca, c	a, f, mi, asp, l	15,290	+	N
36	M	33	AD, AS	42	t, g, w	n, e, mk, s, so, wh, ap, ca, c	a, f, mi, asp, l	25,804	+	Y
37	M	33	AD, AS, RC	38	t, g, w	n, e, mk, ap, ca, c, so, wh	a, f, mi, asp	6,140	+	N
38	F	53	AD, AS, AR	ND		n		35.4	—	N
39	M	38	AD, RC	ND	t, g, w	n, e, mk, s, po, c, ap, wh, so, p	a, f, mi, asp, l, ck	>2,000	+	ND
40	M	30	AD	ND	t, g, w	n, e, wh, so	a, mi, f, l	>5,000	+	Y
41	F	24	AD, AS, RC	ND	t, g, w	n, e, mk, s, wh, so	a, mi	>5,000	+	ND
42	M	56	AD, RC	ND	t, g	n, e, mk, s, ap, c, wh, so, p	a, f, mi, asp, l, ck	1,720	+	ND
43	F	26	AD	ND	g	mk	a, mi	778	—	ND
44	M	39	AD	ND	t, g, w	n	a, f	1,060	—	ND
45	M	16	AD, RC	ND	t, g, w	n	f	398	—	ND
46	F	27	AD, RC	ND	t, g		mi	67.7	—	ND
47	M	35	AD, AS, AR	45	t, g, w	so	a, f, mi, asp	12,499	—	N
48	M	32	AD, AR	44	t			68.7	—	N
49	F	40	AD, AR	24	t, g, w		a, f, mi	1,119	—	N
50	M	72	AD	33	t, g, w		a, f, mi, asp	3,668	—	N
51	F	25	AD, AS, AR	42	t, g, w		a, f, mi, asp	1,498	—	N
52	F	47	AD, AS, AR	31	t, g, w		a, mi, asp	1,020	—	N
53	M	25	AD, AS, AR	31	t, g, w		a, f, mi, asp	2,262	—	Y
54	F	22	AD, AS, AR	61	t, g, w	n, so, mk, s, wh	a, f, mi, asp	66,286	+	Y
55	F	27	AD	22			mi	36	—	N
56	F	29	AD	12	ND	ND	ND	38.5	—	N
57	F	29	AD, AS, AR	13	ND	ND	ND	43.9	—	N
58	M	22	AD, AR	16	t, g		a, mi	1,308	—	N
59	M	40	AD	28			f	2,298	—	N
60	F	37	AD, AS, AR	51	t, g, w	n, so	a, mi, asp	26,200	—	N
61	M	48	AD	30			mi	184	—	N

(Continued)

TABLE E1. (Continued)

No.	Sex	Age (y)	Symptoms	SCORAD	Allergies			Total IgE (kU/L)	FBP IgE	Bacterial infection
					Pollen	Food	Other			
62	F	34	AD	24			a, f, mi	1,310	—	N
63	F	26	AD	52	w		a, f, asp	843	—	Y
64	F	38	AD	51	t, g		a, f, mi, asp	2,661	—	Y
65	M	20	AD, AR	54	g		a, f, mi, asp	5,643	—	Y
66	M	30	AD, AS, AR	20	t, g, w	so	a, f	1,042	—	N
67	M	43	AD, AS, AR	60	t, g, w	e, s, wh, n, so	a, f, mi, asp	15,119	+	N
68	M	62	AD	56	t, g, w	wh, n, so	a, f, mi, asp	11,544	—	N
69	M	49	AD, AS	46	t, g, w		a, f, mi, asp	29,240	+	N
70	F	28	AD, AS, AR	50	t, g, w		a, mi	1,815	—	Y
71	F	46	AD, AS, AR	53	t, g, w		a, f, mi, asp	11,403	—	N
72	M	60	AD, AS, AR	41			a, mi, asp	12,841	—	N
73	F	19	AD	48			a, f, asp	830	—	Y
74	F	23	AD, AR	10	t, g		a, mi	861	—	N
75	M	37	AD	22	ND	ND	ND	13.8	—	N
76	M	25	AD, AR	31	t, g, w		a, f, mi, asp	3,964	—	N
77	M	32	AD, AS	25	t, g, w		a, f, mi, asp	13,113	—	Y
78	F	33	AD, AS, AR	61	t, g	so	f, mi	1,216	—	N
79	F	20	AD	54	ND	ND	ND	5.9	—	Y
80	F	44	AD	47	ND	ND	ND	23.4	—	N
81	M	36	AD, AS	49	t, g, w	k, s, e, mk, wh, n, so	a, f, mi, asp	19,790	+	N
82	M	44	AD, AS, AR	43	t, g, w		a, f, mi, asp	19,545	—	N
83	M	53	AD, AS	62	t, g, w		a, f, mi, asp	21,428	+	N
84	M	34	AD, AS, AR	56	t, g, w		a, f, mi, asp	33,700	+	N
85	F	18	AD, AS, AR	28			mi	31.5	—	N
86	F	39	AD, AS, AR	47	t, g	n	a, f, mi, asp, l	463	—	N
87	F	26	AD, AS, AR	8	t		f	68.5	—	N
88	M	43	AD, AR	49	t, g, w		a, f, mi, asp, l	11,950	+	N
89	F	54	AD, AS, AR	45	g, w		a, f, mi, asp, l	9,400	—	N
90	M	25	AD, AS, AR	63	t, g, w		a, f, mi, asp, l	40,200	+	N
91	M	27	AD	ND	t, g, w	n, ap, p	a	205	—	ND
92	F	43	AD, RC	ND	t, g	n, ap, p, mk	a, mi	19,464	+	ND
93	F	31	AD	ND	t	ap	a	2,923	+	ND
94	M	43	AD, RC	ND	t, g			6,952	+	ND
95	M	ND	AD	ND	t	ND	ND	4,342	+	ND

+, Positive reaction; —, negative reaction; a, animal fur; ap, apple; AS, asthma; asp, *Aspergillus fumigatus*; AR, allergic rhinitis; b, banana; c, celery; ca, carrot; ck, cockroach; e, egg; f, fungus; F, female; g, grass; k, kiwi; l, latex; M, male; mi, mites; mk, milk; n, nuts; N, no; ND, not determined; p, peach; po, potato; RC, rhinoconjunctivitis; s, seafood; SCORAD, Scoring Atopic Dermatitis; so, soy; t, trees; w, weeds; wa, wasp; wh, wheat; Y, yes.

TABLE E2. Demographic, clinical, and serologic characterization of patients with asthma and rhinitis without AD

Patient no.	Sex	Age (y)	Symptoms	Allergies			Total IgE (kU/L)
				Pollen	Food	Other	
AR1	F	35	AR	t	ap, c, n		434
AR2	M	61	AR	t, w, g	n, po, ap, c, so, p	l	119
AR3	M	31	AR			mi	29.6
AR4	M	32	AR	t, g			247
AR5	M	29	AR	t, w, g	n, ap, so, p	mi, l	60.8
AR6	M	33	AR	g			34.2
AR7	M	42	AR	g	po	a	385
AR8	M	23	AR	t, w, g	n, ap, wh, so, p	mi	147
AR9	M	48	AR	t, g	n, c, ap, p	mi	28.6
AR10	F	30	AR	t, g			112
AR11	F	31	AR	t, g		a, mi	45.7
AR12	M	32	AR	t, w, g	n, po, c, wh	asp	77.4
AR13	M	26	AR	g		a, asp, mi	30.3
AR14	F	35	AR	g			168
AR15	M	45	AR	t, g	n, ap, p	a, mi	350
AR16	M	22	AR	t, w, g	n, po, wh, so	mi, l, ck	110
AR17	F	26	AR	t, g		mi	118
AR18	F	25	AR			mi, ck	99.5
AR19	M	22	AR			mi	67.2
AR20	M	26	AR	g		mi	130
AR21	F	63	AR	t, g	n, e, ap, p		ND
AS1	F	55	AS, AR	t, w, g		a, mi	588
AS2	F	20	AS, AR			mi	19.6
AS3	F	28	AS, AR	t, g		mi	146
AS4	M	39	AS, AR	t, g		mi	180
AS5	F	28	AS, AR, U	t, w, g	n, ap	a, f, asp, mi, pen, ck	457
AS6	F	33	AS, AR	t, g	n, e, c, ap, so, p	a, asp	753
AS7	F	67	AS, AR	t, w, g	n, e, ap, so, p	a, f, asp, mi, pen	1830
AS8	F	25	AS, AR	w, g		a, mi	510
AS9	M	26	AS, AR	t, g		a, mi	528
AS10	M	24	AS, AR			mi	540
AS11	M	45	AS			mi	24.9
AS12	M	28	AS, AR	g		a, mi	46.8
AS13	M	36	AS, AR	t, g		a, mi	296
AS14	M	25	AS	g		a, mi	187
AS15	F	26	AS, AR	g		mi	384
AS16	M	24	AS, AR	t, w, g	n	a, mi	522
AS17	M	27	AS, AR	t, g		a, mi	198

a, Animal fur; ap, apple; AS, asthma; asp, *Aspergillus fumigatus*; AR, allergic rhinitis; c, celery; ck, cockroach; e, egg; f, fungus; F, female; g, grass; l, latex; M, male; mi, mites; n, nuts; ND, not determined; p, peach; po, potato; pen, penicillin; so, soy; t, trees; u, urticaria; w, weeds; wh, wheat.

TABLE E3. Entries of amino acid sequences in the GenBank with high similarities to FBP

No.	Accession no.	% Identity	Changes in amino acid	Reference
1	YP_186083	99	L171P	Gill et al, 2005 ^{E6}
2	NP_371732	99	L171P, S565L	Kuroda et al, 2001 ^{E7} ; Ohta et al, 2004 ^{E8}
3	YP_493798	99	L171P, M216I, H302Y	Diep et al, 2006 ^{E9}
4	YP_001246642	99	L171P, R289H, L558H	Unpublished
5	NP_645908	99	E88K, D102E, L171P	Baba et al, 2002 ^{E10}
6	YP_040595	99	I169M, L171P, S211N, L558H	Holden et al, 2004 ^{E11}
7	ZP_04018173	99	D111N, I169M, L171P, S211N, L558H	Unpublished
8	YP_416554	98	I169M, L171P, S211N, H244N, Q258H, A441E, L558H	Herron-Olson et al, 2007 ^{E12}

TABLE E4. Percent pairwise sequence similarities between amino acid sequences of 6 bacterial FBPs*

	Similarity (%)					
	Staa	Step	Lism	Clod	Fusn	Borb
Staa	100.0	39.3	42.7	38.6	28.0	24.4
Step		100.0	44.5	34.4	27.8	21.5
Lism			100.0	40.4	26.6	23.2
Clod				100.0	30.0	24.3
Fusn					100.0	19.1
Borb						100.0

**Staphylococcus aureus* (Staa), *Streptococcus pneumoniae* (Step; Accession number ZP_01408679.1), *Listeria monocytogenes* (Lism; NP_465354.1), *Clostridium difficile* (Clod; AAM73699.1), *Fusobacterium nucleatum* (Fusn; NP_603579.1), *Borrelia burgdorferi* (Borb; NP_212481.1).