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SUPPORTING INFORMATION

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IgE reactivity against herpes simplex virus 1 in patients with atopic dermatitis complicated by eczema herpeticum

To the Editor,

Eczema herpeticum (EH) is a severe and widespread viral skin infection caused by herpes simplex virus 1 (HSV-1), which particularly affects patients with atopic dermatitis (AD) who have a more severe course of the disease, higher allergen sensitization, and greater Th2 polarization.^{1,2} The condition has been named ADEH (AD complicated by EH). The pronounced shift to a Th2 immune response in ADEH has been proposed as a cause of an ineffective immune response against microorganisms.³ Th2 cell predominance goes along with overproduction of cytokines such as IL-4 and IL-13 which are key for IgE induction and may prevent the development

of cellular immunity against microorganisms. Specific IgE can be produced against microorganisms such as bacteria and yeasts in a subgroup of patients with AD with a potential contribution to AD severity.^{4,5} Evidence also exists that IgE can be produced against viruses.^{6,7,51-53} Although the role of specific IgE in viral infections is still unknown, this might be associated with exacerbation of atopic diseases.^{8,9,54-55} In humans, specific IgE against different viruses has been linked with poorer prognosis.^{52,53,56} In that respect, it has been suggested that virus-specific IgE should be analyzed due to its possible association with atopic diseases.⁵⁴ Currently, there is only sparse information about IgE-mediated mechanisms against viruses

such as HSV-1 in inflammatory skin diseases with a Th2 predominance such as ADEH.

In the present study, an ELISA was developed to detect specific IgE against HSV-1 in the sera of patients with ADEH, AD, and healthy controls. IgG against HSV-1 was also analyzed in the sera from the same patients and controls. We analyzed immediate type I hypersensitivity response to purified and lysed HSV-1, from direct stimulation of IgE-bearing cells, such as basophils by means of basophil activation test (BAT). Proliferative response in a lymphocyte transformation test (LTT) and cytokine production after exposure to purified and lysed HSV-1 were also investigated. IgE-binding proteins from HSV-1 were analyzed by IgE-Western blot, identified by mass spectrometry and recombinantly produced. A mouse model (*Mus musculus*, BALB/c) of epicutaneous sensitization (EC) was used to study the allergenic potential of IgE-binding

proteins from HSV-1 (clinical characteristics of patients and controls is shown in Table S1. Full description of methods can be found in the Data S1).

Specific IgE against HSV-1 could be detected in most of the sera from ADEH patients, with OD values above the cutoff point of positivity for most of them. Levels of specific IgE against HSV-1 were higher in the sera of ADEH patients compared to AD patients ($P < 0.001$) and healthy controls ($P < 0.001$) (Figure 1A). Experiments of sera dilution showed a dose-response relationship that demonstrates that the binding of IgE is specific to HSV-1 (Figure S1). Additionally, IgE against HSV-1 correlated with IgG against HSV-1 in AD patients without EH ($P < 0.05$) (Figure S2B) and in healthy controls ($P < 0.001$) (Figure S2C). In ADEH, there is also a positive correlation although with a P -value of 0.06 (Figure S2A).

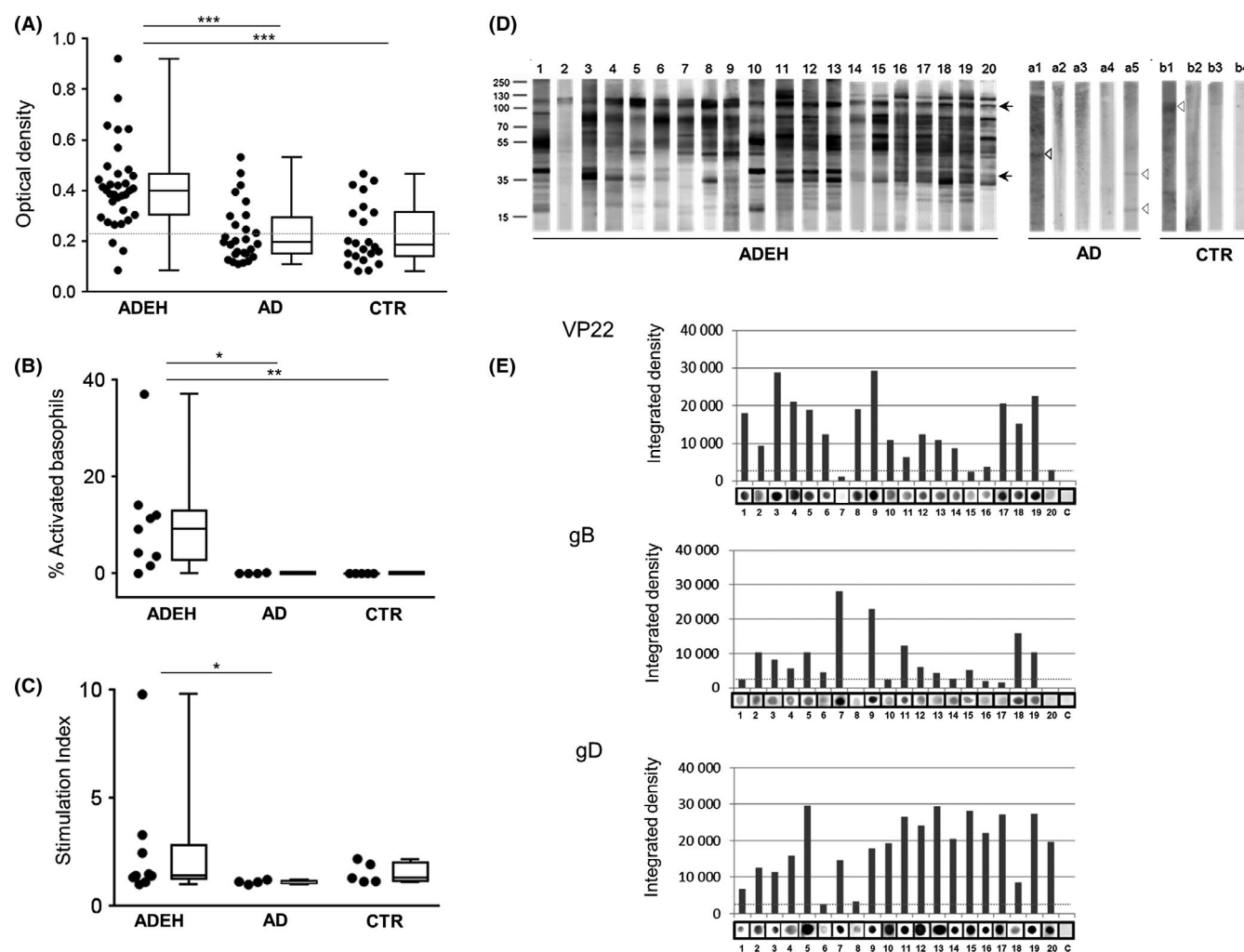


FIGURE 1 A, Analysis of specific IgE against HSV-1 in sera from ADEH, AD patients, and healthy controls by ELISA. The dashed horizontal line marks the cutoff point of positivity (0.225). B, Percentages of activated basophils (CD63+ CD203c+) after subtracting basal degranulation in BAT upon stimulation with purified and lysed HSV-1. C, Stimulation index obtained in LTT after stimulation with purified and lysed HSV-1. The results are shown in box-and-whisker plots. Each data group is accompanied by a scatter dot plot of the same results. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. D, IgE-Western blot of HSV-1 proteins transferred to membranes and incubated with sera from a subset of ADEH patients. As control, membranes were incubated with the sera from a subset of AD patients and healthy subjects randomly chosen. The IgE-binding proteins of 120 and 41 kDa selected for mass spectrometry identification are marked with black arrows. Open white arrows mark the few faint bands recognized by controls. E, IgE-dot blot of recombinant VP22, gB, and a third HSV-1 protein, gD, using ADEH sera

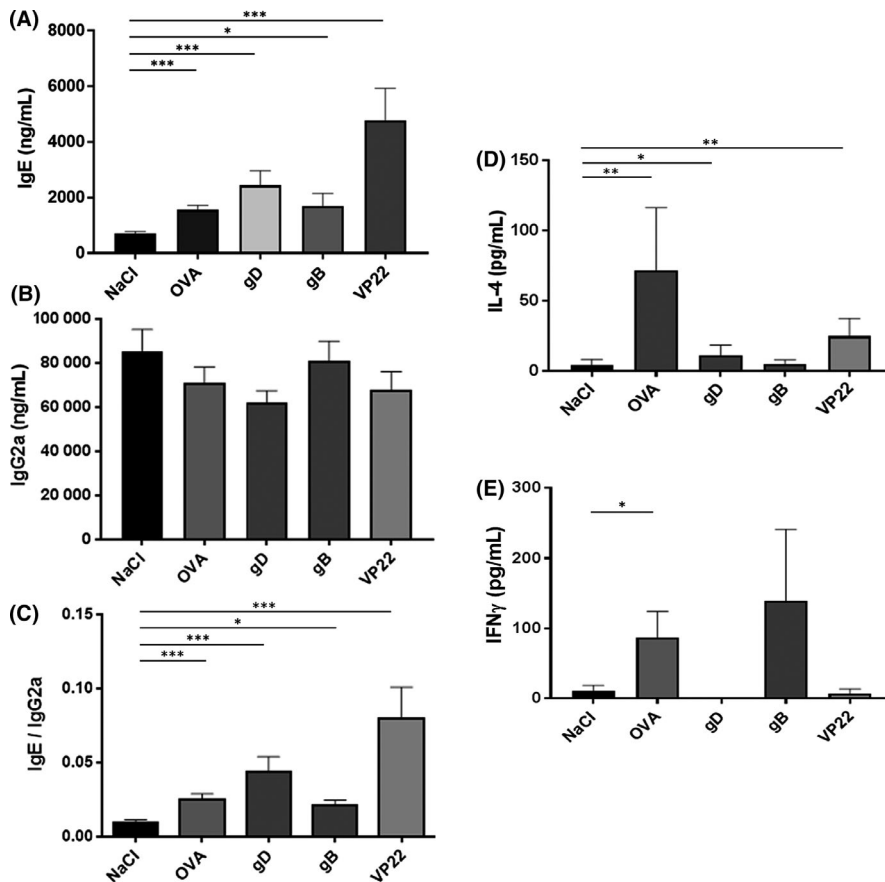


FIGURE 2 A, B, C, Total serum IgE, IgG2a, and IgE/IgG2a ratios, respectively, from mice EC-sensitized with 0.9% NaCl, OVA, gD, gB, and VP22 ($n = 12$ for each group). The detection was carried out by means of ELISA. D, E, Synthesis of IL-4 and IFN- γ , respectively, from single-cell suspensions from skin-draining brachial lymph nodes of mice EC-sensitized with 0.9% NaCl, OVA, gD, gB, and VP22 ($n = 12$ for each group). The detection of IL-4 and IFN- γ in supernatants was carried out by means of ELISA. Data are reported as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Basophil activation test was performed with the whole blood of a subset of ADEH patients, AD patients, and healthy controls randomly chosen. Increased basophil activation was observed in ADEH compared to AD patients ($P < 0.05$) or healthy controls ($P < 0.01$) upon HSV-1 exposure (Figure 1B). Experiments with the RBL-48 cell line underlined the specific role of IgE in the activation of basophils after HSV-1 exposure, although with a modest effect (Data S1, and Figure S3).

We analyzed activation and proliferation of memory T cells after stimulation with purified and lysed HSV-1. LTT was performed with PBMCs from a subset of ADEH patients, AD patients, and healthy subjects randomly chosen. The analysis of the proliferative response revealed a higher stimulation index ($P < 0.05$) in ADEH compared to AD patients (Figure 1C). Furthermore, HSV-1 was able to induce a statistically significant increase in the production of the Th2 cytokine IL-4 in ADEH compared to AD patients but not in the other cytokines tested (Figure S4).

We next aimed to characterize the proteins from HSV-1 recognized by serum IgE from ADEH patients. A complex pattern of IgE-reactive proteins in HSV-1 ranging from 17 to 130 kDa was found in IgE-Western blot (Figure 1D). Proteins with molecular weights of 120, 80, and 41 kDa, among others, were recognized by IgE of more than 50% of the ADEH patients sera (Figure 1D). We focused on two of the IgE-binding proteins from HSV-1 (120 and 41 kDa) for which there was a good separation of the bands with the corresponding molecular weight in the polyacrylamide gel and they were identified

by mass spectrometry as glycoprotein B (gB) (120 kDa) and viral protein 22 (VP22) (41 kDa) from HSV-1 (data not shown). The proteins VP22 and gB (domain I) were recombinantly produced and tested together to a third HSV-1 protein, glycoprotein D (gD) (two immunodominant regions that cover 66% of the sequence), commercially available, in IgE-dot blot using ADEH sera (further details of the recombinant proteins are provided in the Data S1). Figure 1E shows that the recombinant proteins were recognized by IgE of more than 50% of the patients' sera in the IgE-dot blot. This recognition was stronger for gD and VP22 than gB.

In order to investigate whether the recombinant IgE-binding proteins from HSV-1 (gD, gB, and VP22) were able to elicit a Th2 response in vivo, an established mouse model of EC sensitization was utilized. In this model, repeated EC sensitization of tape-stripped skin with allergenic proteins induces local and systemic Th2 inflammation.⁵⁷⁻⁵¹⁰ The results showed that sensitization of mice with the IgE-binding proteins from HSV-1 induced a significant increase in total IgE. The increase was particularly pronounced for mice sensitized with gD and VP22 compared with gB (Figure 2A). Interestingly, although a slight decrease in total IgG2a was achieved in mice sensitized with gD and VP22, the IgE/IgG2a ratios confirmed that EC sensitization with gD and VP22 triggers a robust production of IgE (Figure 2B,C). Furthermore, mice sensitized with gD and VP22 showed a significantly increased secretion of IL-4 but not IFN- γ in single-cell suspensions from skin-draining brachial lymph nodes (Figure 2D, E).

Our study is the first to show specific IgE against HSV-1 in ADEH patients sera and it provides preliminary evidence of an increased type I and type IV hypersensitivity response to HSV-1 in these patients. Our results also showed that although the presence of IgE against HSV-1 seems to be generally related to the exposure to HSV-1 (evidenced by IgG against HSV-1), in the specific case of ADEH, the increased levels of IgE against HSV-1 obtained in this study and showed in Figure 1A seem to be related not only just to a HSV-1 exposure, but also maybe to other factors such as the Th2 polarization characteristic of ADEH patients. Further research will be necessary to deepen this aspect. Previous studies in the late eighties failed to demonstrate IgE against HSV-1 in a study population of seven ADEH patients;⁵¹¹ however, IgE against HSV-1 has been detected in the sera from patients with diseases such as Meniere's disease.⁵¹²

Our findings suggest that in a context of a Th2 cell predominance, which is characteristic for ADEH, IgE against HSV-1 can be produced, with an increased activation of effectors cells of allergy and T cells. Some of the main IgE-binding proteins from HSV-1 have been characterized in this study. A major allergen has been defined as one to which more than 50% of the sera of patients containing specific IgE to a specific allergenic source react.⁵¹³ Here, we showed that especially the viral proteins gD and VP22 were widely recognized by IgE from the ADEH patients sera. Therefore, these proteins seem to be major allergens in HSV-1. gD and VP22 were also able to mount a Th2 immune response in mice, which support the allergic potential of these two viral proteins. Additional studies, however, will be necessary in human samples to confirm these results. Previous studies in mice have also demonstrated that viral components can induce IgE production and they can have an impact on the severity of atopic diseases such as asthma.^{8,9}

Our findings contribute to the growing scientific evidence that IgE against viruses can be made as part of the antiviral response in some atopic diseases, in this case, ADEH. However, further research will be necessary in order to establish the functional relevance of our findings in clinical samples and their potential impact in the severity of ADEH.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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METHODS

Patients and detection of specific IgE against HSV-1 in sera by ELISA

The detection of specific IgE against HSV-1 was performed in the serum of 34 ADEH patients, 27 AD patients and 22 healthy controls. Patients and controls were recruited at Department of Dermatology and Allergy, University of Bonn. AD was diagnosed according to the criteria of Hanifin and Rajka. ADEH patients were defined as subjects with AD who had at least one EH episode. Healthy controls were defined as subjects without personal history of atopic diseases and no personal history of chronic skin or systemic diseases. Neither AD subjects nor healthy controls had evidence of previous HSV-1 infections. Clinical characteristics of patients and controls are shown in Table S1. The study was approved by the Ethics Committee of the University of Bonn (Nr 064/17). Written informed consent was obtained from all participants. The detection of specific IgE against HSV-1 was carried out by means of a modified HSV-1 ELISA kit, which contains plates precoated with total antigens from HSV-1 (Ridascreen, R-Biopharm, Darmstadt, Germany). Sera samples were added (undiluted, 100 µl) in duplicates to the precoated wells and incubated during 2 h at 37 °C. After washing (wash buffer provided with the kit Ridascreen, R-Biopharm, Darmstadt, Germany), wells were incubated with a monoclonal mouse anti-human IgE antibody (clone 1A2, Abbiotec, San Diego, CA, USA) (stock: 1 mg/ml, used at 1:1000) for 1 h at 37 °C. Wells were then washed and incubated with HRP-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Dallas, TX, USA) (stock: 0.4 mg/ml, used at 1:1000) for 1 h at 37 °C. After washing, the peroxidase reaction was developed with tetramethylbenzidine / H₂O₂. The reaction was stopped with 0.5 M sulphuric acid. Optical density (OD) was measured at 450 nm using a Synergy HT multi-detection microplate reader (BioTek, Bad Friedrichshall, Germany). The following negative control was included: HSV-1 precoated-wells incubated with the sample buffer provided with the kit (instead of patient's serum) and then incubated with mouse anti-human IgE and followed by incubation with goat anti-mouse IgG-HRP. All the tests were performed in duplicates. The formula mean [OD] + 3 × SD was calculated for the negative control to establish the cut-off point of positivity.

An additional experiment was carried out in order to demonstrate that the binding of IgE to HSV-1 shows a dose-response curve. In that respect, an analysis of specific IgE against HSV-1 in representative sera from each group (ADEH, AD, and CTR) using undiluted sera and sera diluted 1/4 and 1/8 was carried out. The ELISA was performed similarly as the protocol described for the detection of specific IgE against HSV-1 in sera (previous paragraph) but using in this case undiluted and diluted sera.

Detection of specific IgG against HSV-1 in sera by ELISA

Wells coated with HSV-1 proteins (Ridascreen, R-Biopharm, Darmstadt, Germany) were incubated with the sera from 34 ADEH, 27 AD patients and 22 healthy subjects (1:50 dilution), during 2 h at 37 °C. After washing, an incubation with mouse anti-human IgG antibody (Acris abs. SM3062P, Herford, Germany) (1:5000) was carried out for 1 h at 37 °C. Wells were washed and then incubated with HRP-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Dallas, TX, USA) (1:5000) for 1 h at 37 °C. After washing, the peroxidase reaction was developed with tetramethylbenzidine / H₂O₂ and stopped with 0.5 M sulphuric acid. The OD was measured similarly as described above and similar replicates and negative control as described above were included.

Type I hypersensitivity response from direct stimulation of IgE-bearing cells with HSV-1: Basophil activation test (BAT)

BAT was carried out with the whole blood of a subgroup of ADEH patients (n = 9), AD patients (n = 4) and healthy controls (n = 5) randomly chosen (Flow 2 Cast kit, Böhler Laboratories, Schönenbuch, Switzerland). The test is designed to determine the percentages of activated basophils (CD63⁺ CD203c⁺) from the total number of basophils (CCR3⁺) in whole blood by flow cytometry upon antigen stimulation, in this case commercially available purified and lysed HSV-1 at 100 µg/ml. The purified and lysed HSV-1 was obtained as follows (Zeptomatrix, Buffalo, NY, USA): HSV-1 was propagated in Vero cells. At the appropriate time infected cells were disrupted to release virus particles by freeze thaw and sonication. Virus particles released from cells were partially purified by low speed centrifugation to remove cell debris. HSV-1 particles were then purified by sucrose density gradients. The purified virus was then pelleted again and resuspended in disruption buffer to generate the lysate. The lysate contains all the viral components, including all its antigens. For BAT, a monoclonal anti-FcεRI antibody and N-formyl-methionyl-leucyl-phenylalanine were utilized as positive controls. The evaluation of the basal degranulation was performed by using buffer alone (the buffer in which HSV-1 is resuspended). The mix of staining reagents included monoclonal antibodies to human CD63 and CD203c labeled with the tandem dye PE-DY647, and a monoclonal antibody to CCR3 (CD193) labeled with PE. The percentages of activated basophils upon stimulation with purified and lysed HSV-1 were analyzed by flow cytometry according to manufacturer instructions.

RBL-48 experiments

The basophilic cell line RBL-48 transfected with the human IgE receptor FcεRIα (a gift from Dr. J. Kochan)^{S14} was used in order to analyze the role of IgE in the activation of basophils after HSV-1 exposure. RBL cells were cultured in very low endotoxin RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% antibiotic/antimycotics and 500 µg/ml of active geneticin (Merck Chemicals, Nottingham, UK). The expression of the FcεRIα was confirmed by flow cytometry, using an anti-human FcεRIα subunit antibody (eBioscience Inc, San Diego, CA, USA). Fifty µl of cells at 2 x 10⁶/ml were plated in a 96-well culture plate (Corning Inc, NY, USA) and passively sensitized by incubation with 5 individual sera from ADEH patients with positive IgE anti-HSV-1 (final dilution 1:20) at 37 °C, 5% CO₂ overnight. Additionally, a pre-incubation step of the ADEH sera with an IgE neutralizing antibody (anti-IgE monoclonal antibody E25, Novartis) for 2h 30 min before cell loading was also included in the experiment in order to block the binding of IgE from the sera to RBL cells. Then, RBL cells sensitized with sera or IgE-neutralized sera were exposed in triplicate for 60 min to HSV-1 or a rabbit anti-human IgE cross-linking stimulus (polyclonal antibody, code A0094, Agilent Dako) (as a positive control of IgE cross-linking). β-hexosaminidase release was measured as previously described.^{S15}

Lymphocyte transformation test (LTT) and production of Th2/Th1 cytokines

LTT was performed with unfractionated PBMCs population^{S16} freshly isolated from the blood of a subgroup of ADEH patients (n = 9), AD patients (n = 4) and healthy subjects (n = 5) randomly chosen and subjected to density gradient. Cultures were established in triplicate in 96-well plates in the presence of purified and lysed HSV-1 or buffer alone (the buffer in which HSV-1 is resuspended). After 72 hours, the cell proliferation was determined by XTT

Cell Proliferation Kit (PromoKine, Heidelberg, Germany). The stimulation index was calculated as mean of proliferation of triplicate cultures stimulated with purified and lysed HSV-1 divided by mean of proliferation of triplicate cultures stimulated with the buffer in which HSV-1 is resuspended. Parallel PBMCs cultures were established and stimulated in the same way but during 8 days and the supernatants were recovered for cytokine measurement (IL-4, IL-13, IL-5, IL-10, TNF- α , and IFN- γ) using a cytometric bead immunoassay (CBA Flex Set system, BD Biosciences, Heidelberg, Germany).

Characterization of IgE-binding proteins from HSV-1 by IgE-western blot and identification by mass spectrometry

In order to characterize the proteins from HSV-1 recognized by serum IgE from ADEH patients, a western blot analysis was carried out with purified and lysed HSV-1. Six μ g of HSV-1 lysate were mixed with Laemmli sample buffer and β -mercaptoethanol, heated at 95 °C for 10 min, and electrophoresed in a 10 % Tris-HCl gel. The resolved HSV-1 proteins in gel were transferred to polyvinylidene difluoride membranes (PVDF) (Merck, Darmstadt, Germany). Membranes were cut into 3-mm strips and then blocked with tris-buffered saline containing 0.5% tween-20 and 5 % non-fat milk for 1 h at room temperature (blocking solution). The strips were incubated overnight with individual sera (1:10 dilution) from a subset of 20 ADEH patients, 5 AD patients, and 4 healthy controls, washed (in tris-buffered saline containing 0.5% tween-20), and then treated with a monoclonal mouse anti-human IgE antibody (clone 1A2, Abbiotec, San Diego, CA USA) (stock: 1 mg/ml, used at 1:1000) for 3 h at room temperature. Strips were washed and incubated with HRP-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Dallas, TX, USA) (stock: 0.4 mg/ml, used at 1:1000) for 1 h at RT. After washing, the detection was achieved by enhanced chemiluminescence, according to the instructions of the manufacturer (Amersham Biosciences, Piscataway, NJ, USA). We next aimed to identify the main IgE-binding proteins from HSV-1 by means of mass spectrometry analysis. We selected two of the main IgE-binding proteins (120 and 41 kDa proteins) and the bands with the corresponding molecular weights were excised from a coomassie blue-stained polyacrylamide gel in which an appropriate separation of the HSV-1 proteins was carried out for this analysis. In-gel trypsin digestion was performed and the digests were analysed via mass spectrometry as previously described.^{S17}

Recombinant production of IgE-binding proteins from HSV-1 and analysis in IgE-dot blot

The recombinant proteins VP22 and gB (domain I) were custom produced by Virogen (Watertown, MA, USA) as specified below. gD was commercially available.

- For VP22 the whole sequence was considered in order to produce the recombinant protein. The molecular parameters of the recombinant protein are the following: PI/MW: 10.5 / 33,500.

- For gB (domain I). The domain I of gB was considered in the production of the recombinant protein, since according to Virogen the full sequence was difficult to obtain recombinantly

due to its large molecular weight. Therefore, we selected for recombinant production domain I of gB because preliminary data of IgE-epitope mapping considering the full sequence of gB showed that the domain I contains several IgE-binding epitopes (unpublished data). The molecular parameters of the recombinant protein (domain I) are the following: PI/MW: 8.36 / 24,507

- Recombinant gD was commercially available (Virogen, Watertown, MA, USA). As specified by the company it contains two immunodominant regions of gD that covers 66 % of the sequence (region 1: from amino acid 84 to amino acid 175 and region 2: from amino acid 226 to amino acid 394). The molecular parameters of the recombinant protein are the following: PI/MW gD region 1: 6.39 / 10,325. PI/MW gD region 2: 5.92 / 18,414

The proteins were expressed in *E.coli* by Virogen (Watertown, MA, USA). Briefly, the antigens were de novo synthesized and cloned into pET24 b+ *E. coli* expression vector, Nde I / Xho I sites. The proteins were induced overnight at RT with IPTG, 0.5 mM. Cells were harvested and lysed with *E.coli* lysis buffer, centrifuged and supernatant was passed over a Ni-Agarose column. Proteins were washed with 10 mM imidazole buffer and eluted in 8 M Urea, PBS1 X, 0.5 M Imidazole. Fractions containing the antigen were concentrated in the Pierce 10 kDa MW cut off concentrators and passages through the G-50 desalting columns. Concentration was measured by Nanodrop at A280. The purity of all the recombinant proteins were > 90%.

IgE-dot blot was performed by applying recombinant proteins (2 µg protein per dot) on PVDF membranes, blocking in blocking solution, and incubating overnight with individual sera (1:10 dilution). The rest of the assay was performed as described above for IgE-Western blot. The integrated density of each dot was measured using Image J software (NIH, Bethesda, MD, USA). Integrated density values lower than 2500 were considered as background negative signal.

Experiments with mice

Sensitization of mice with IgE-binding proteins from HSV-1

Female BALB/c mice (*Mus musculus*) obtained from Charles River (Sulzfeld, Germany) were kept in a pathogen-free environment in the animal facility of the LIMES Institute, University of Bonn, Germany. Mice were maintained under conventional laboratory conditions (22°C, 12 hr/ 12 hr light/dark cycle) in standard animal cages and they were used for experiments at age 6 weeks. Permission of the government of North Rhine-Westphalia was obtained for these experiments (Az. 84-02.04.2014.A437). Isoflurane anesthetized mice were shaved on the back and tape-stripped three times with transparent dressing Tegaderm 1624W (3M, Neuss, Germany). Ovalbumin (OVA) (positive control, 100 µg in 0.9% NaCl) (endotoxin-free, Hyglos, Bernried, Germany), gD, gB, VP22 (50 µg in 0.9% NaCl) (endotoxin-free, Virogen, Watertown, MA, USA), or 0.9% NaCl alone (negative control) were placed on a sterile gauze patch (1 x 1 cm) and fixed to the skin with Tegaderm 1624W. The application of the patches was performed on days 1 and 4 and they were kept of a total of 1 week. Then, after a 2-week rest, mice were subjected to tape-stripping again and new patches were applied in the same place. Mice had a total of three 1-week exposures to the patches, separated by 2-week intervals from each other. In the third cycle, an additional tape-stripping and patch application was carried out on day 6. In total, there were 12 mice per group.

Quantification of total serum IgE and IgG2a

The collection of sera was carried out at the end of the period of sensitization (day 49). Total IgE was quantified by ELISA using Mouse IgE ELISA MAXTM standard set (BioLegend, Germany). Wells were coated with capture antibody (anti-mouse IgE ELISA MAX, capture antibody, BioLegend, Germany) overnight at 4°C. Wells were then washed and blocked with assay diluent (BioLegend, Germany) for 1 hour. After washing, diluted mouse IgE standard (BioLegend, Germany) and sera samples were added to the appropriated wells and incubated at room temperature for 2 hours. Detection antibody (biotinylated anti-mouse IgE ELISA MAX detection antibody, BioLegend, Germany) was added after washing and incubated at room temperature for 1 hour. Wells were washed and avidin-HRP solution was added and incubated at room temperature for 30 minutes. After that, wells were washed and soaked for 1 minute per wash, and TMB substrate solution was added to each well and incubated in the dark for 20-30 minutes. The reaction was stopped with 2N sulphuric acid. OD was measured at 450 nm using a Synergy HT multi-detection microplate reader (BioTek, Bad Friedrichshall, Germany). Total IgG2a in sera was quantified with Mouse IgG2a ELISA set (BD Biosciences, Heidelberg, Germany). The protocol for IgG2a quantification was similar to the one described for total IgE but using an anti-mouse IgG2a as capture antibody, a biotinylated anti-mouse IgG2a as detection antibody and mouse IgG2a as standard (BD Biosciences, Heidelberg, Germany). All the tests were performed in duplicates.

Determination of IL-4 and IFN-γ synthesis in lymph nodes

Skin-draining brachial lymph nodes were harvested at the end of the period of sensitization (day 49). Single-cell suspensions were obtained from the lymph nodes after incubation with collagenase D (1 mg/ml) and DNaseI (300 U/ml) for 15 minutes at 37°C. Cells were seeded in 96 well plates in duplicates and restimulated for 3 days with OVA (100 µg/ml) (endotoxin-free, Hyglos, Bernried, Germany), gD, gB, VP22 (50 µg/ ml) (endotoxin-free, Virogen, Watertown, MA, USA), or 0.9% NaCl. Supernatants were collected and analyzed for IL-4 and IFN-γ production by means of ELISA (Mouse IL-4 and Mouse IFN-γ ELISA MAXTM standard set, BioLegend, Germany). In brief, wells were coated with capture antibody overnight (anti-mouse IL-4 or anti-mouse IFN-γ ELISA MAX, capture antibody, BioLegend, Germany). Diluted standards (mouse IL-4 or mouse IFN-γ) and samples were added to the appropriated wells after blocking. Bound IL-4 or IFN-γ were detected with detection antibodies (biotinylated anti-mouse IL-4 or IFN-γ ELISA MAX detection antibodies, BioLegend, Germany). Avidin-HRP solution was added and the rest of incubations and the method of detection are similar as described in the previous section. All the tests were performed in duplicates.

Statistical analysis

The Mann-Whitney U test was used for statistical analysis. Spearman's rank correlation test was used to determine correlations. Differences were considered significant with $P < 0.05$. The statistics software GraphPad Prism version 7 for Windows (GraphPAD Software, San Diego, CA, USA) was used.

RESULTS

RBL-48 experiments

In order to elucidate the role of IgE in the activation of basophils after HSV-1 exposure, we performed an experiment with a basophilic cell line RBL-48. In this experiment, RBL-48 cell line expressing the human high-affinity IgE receptor FcεRI was loaded with individual sera from 5 ADEH patients with positive IgE anti-HSV-1 and was exposed to HSV-1 or a rabbit anti-human IgE cross-linking stimulus (as a positive control of IgE cross-linking). Additionally, a pre-incubation step of the sera with an IgE neutralizing antibody (anti-IgE) before cell loading was also included in the experiment in order to block the binding of IgE from the sera to RBL cells previous to the exposure to HSV-1 or the rabbit anti-human IgE cross-linking stimulus. Then the release of the allergic mediator β -hexosaminidase, which is also a marker of basophil activation, was measured in triplicate. The results showed that the neutralization step with anti-IgE (that blocks the binding of IgE from the sera to the cells) decreased the release of β -hexosaminidase when they were exposed to HSV-1 ($p < 0.05$) and to the IgE cross-linking stimulus ($p < 0.01$) when compared to the release of cells fully loaded with IgE (without IgE neutralization step) and exposed to HSV-1 or the IgE cross-linking stimulus (Figure S3). In that way, the results suggest that the activation and release of allergic mediators by basophils are mediated by the recognition of HSV-1 by IgE anti-HSV-1, although with a modest effect. The results also suggest that the presence of HSV-1 alone cannot explain the triggering of β -hexosaminidase release. The presence of both IgE anti-HSV-1 and the virus HSV-1 was necessary.

FIGURES

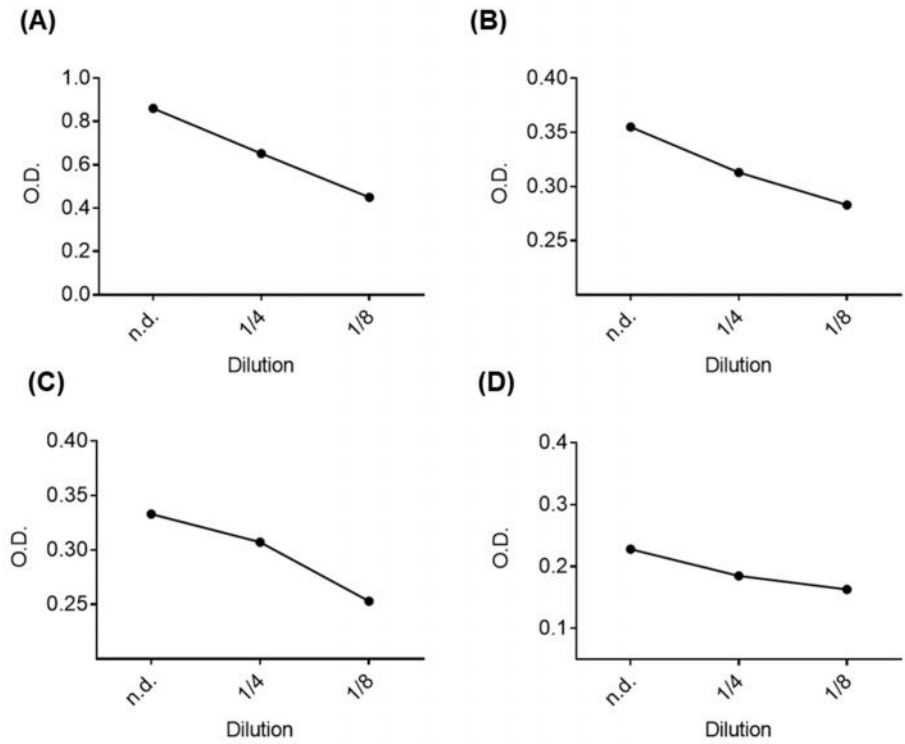


Fig S1. Analysis of specific IgE against HSV-1 in sera from two ADEH patients (graphs A and B), one AD patient (graph C), and one healthy control (graph D) using not diluted sera (n.d.), and sera diluted 1/4 and 1/8. The ELISA was carried out similarly as described for the detection of specific IgE against HSV-1 in sera (in the section methods in supporting information) but using in this case undiluted and diluted sera.

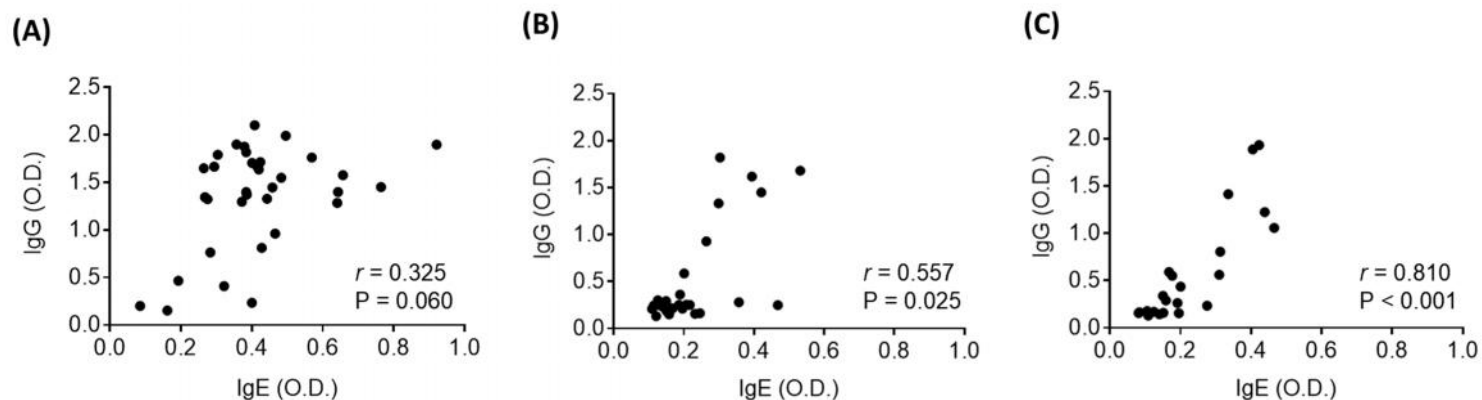
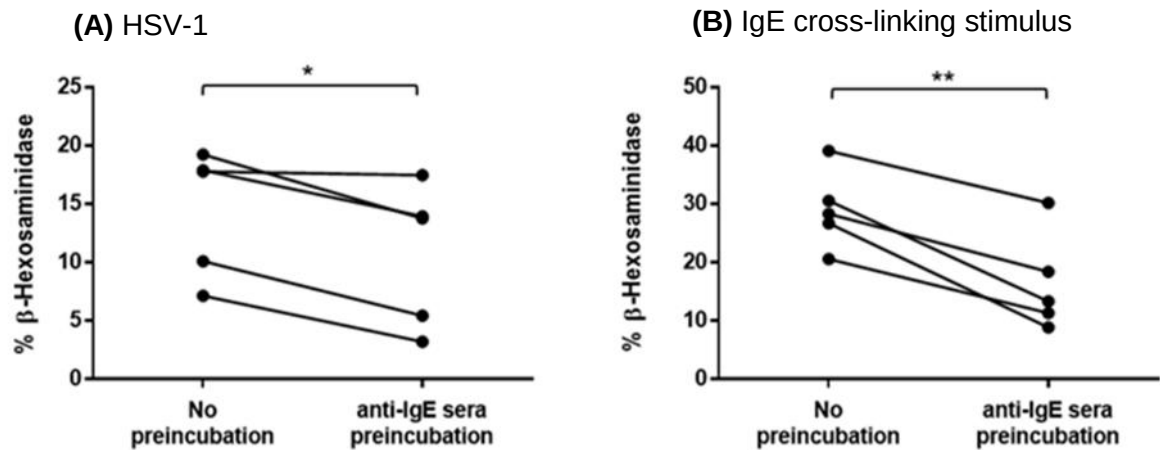


Fig S2. Correlation between specific IgG against HSV-1 and specific IgE against HSV-1 detected in the sera of ADEH patients (A), AD patients (B), and healthy controls (C). Spearman's rank correlations coefficients and significance levels are shown in the figures.

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Figure S3. IgE-mediated release of β -hexosaminidase from RBL-48 by HSV-1. RBL cells expressing human Fc ϵ RI were loaded with individual sera from 5 ADEH patients containing specific IgE against HSV-1. The serum was pre-incubated with or without IgE-neutralizing antibody before cell loading, indicated in the graphs as anti-IgE sera preincubation or no preincubation respectively. Cells were then exposed to HSV-1 (A) or a rabbit anti-human IgE cross-linking stimulus (B). Percentages of total β -hexosaminidase release are shown. Statistical analyses were performed using a two-tailed, paired t-test.

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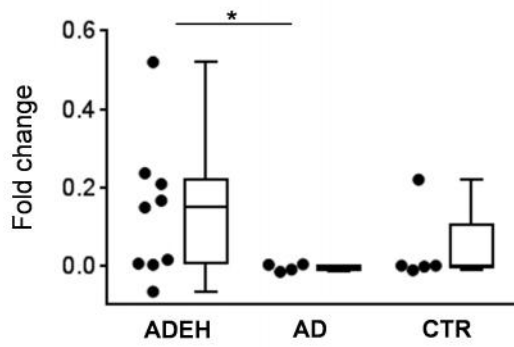
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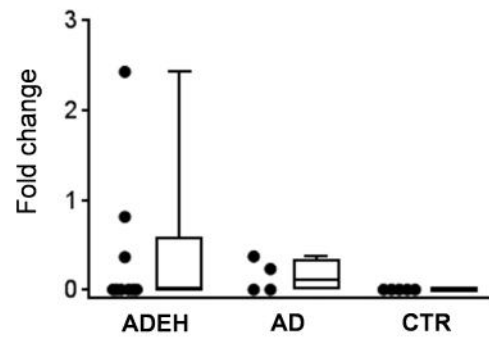
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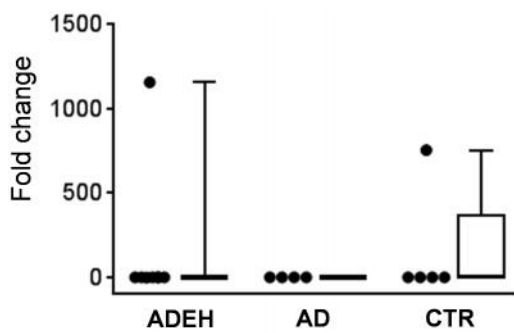
IL-4



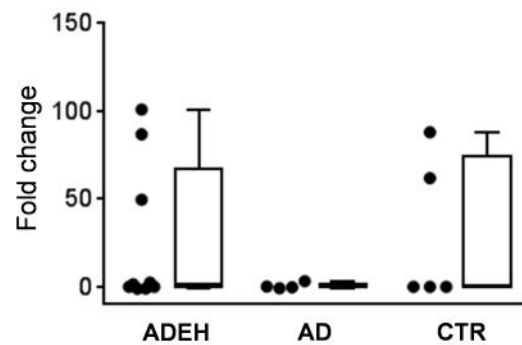
IL-13



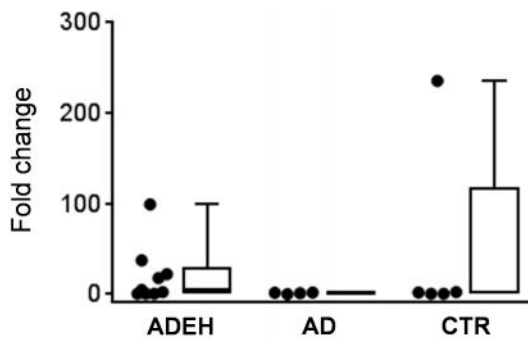
IL-5



IL-10



IFN- γ



TNF- α

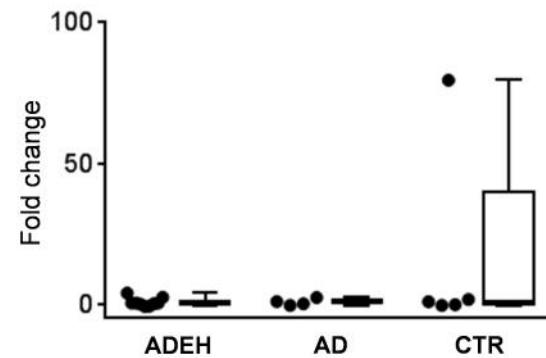


Fig S4. Cytokine production upon HSV-1 stimulation. PBMCs cultures were established in the presence of purified and lysed HSV-1 or buffer alone. After 8 days of culture, supernatants were recovered for cytokine measurement. Fold change in cytokine concentration was calculated as $(A-B)/B$, being A cytokine concentration in culture stimulated with HSV-1 lysate and B cytokine concentration in culture stimulated with buffer alone. * $P < 0.05$

TABLE S1. Clinical characteristics of patients and controls

	ADEH	AD	CTR
Sample size	34	27	22
Males (%)	73	33	36
Age (y)	34.2	33.5	41.9
Total IgE (IU/mL)	6092.7	4663.3	42.6
SCORAD	38.4	29.8	0

Supplementary References

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