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A Soluble Form of the High Affinity IgE Receptor, Fc-Epsilon-RI, Circulates in Human Serum

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Abstract

Soluble IgE receptors are potential in vivo modulators of IgE-mediated immune responses and are thus important for our basic understanding of allergic responses. We here characterize a novel soluble version of the IgE-binding alpha-chain of Fc-epstein-RI (sFcεRI), the high affinity receptor for IgE. sFcεRI immunoprecipitates as a protein of ~40 kDa and contains an intact IgE-binding site. In human serum, sFcεRI is found as a soluble free IgE receptor as well as a complex with IgE. Using a newly established ELISA, we show that serum sFcεRI levels correlate with serum IgE in patients with elevated IgE. We also show that serum of individuals with normal IgE levels can be found to contain high levels of sFcεRI. After IgE-antigen-mediated crosslinking of surface FcεRI, we detect sFcεRI in the exosome-depleted, soluble fraction of cell culture supernatants. We further show that sFcεRI can block binding of IgE to FcεRII expressed at the cell surface. In summary, we here describe the alpha-chain of FcεRI as a circulating soluble IgE receptor isoform in human serum.

Introduction

Allergic patients are commonly characterized by high serum IgE and high IgE-receptor expression on effector cells of the innate and adaptive immune system [1,2]. In humans, three different IgE-receptors have been described: CD23, galectin-3 and FcεRI [1,2]. CD23, also known as FcεRII, is a low affinity IgE receptor and the classical IgE receptor on B cells. Galectin-3, formerly known as epsilon binding protein (εBP), is another low affinity IgE receptor; its role in allergy is rather poorly defined [3,4]. FcεRI, the high-affinity receptor for IgE, induces activation of mast cells and basophils via IgE-antigen complexes during the acute phase of an allergic response [5,6]. In rodents, FcεRI is constitutively expressed on the surface of basophils and mast cells as a tetrameric receptor composed of the ligand-binding alpha-chain, one beta-chain and a pair of disulfide-linked gamma-chains. Humans can express a trimeric version of FcεRI lacking the beta-chain on eosinophils and antigen presenting cells, such as dendritic cells and Langerhans cells [6,7]. Additionally, expression of FcεRI on bronchial and intestinal epithelial cells was described in humans [8,9]. Serum IgE binding stabilizes surface FcεRI leading to the upregulation of receptor levels in allergic patients [10,11,12].

In contrast to the transmembrane forms, CD23 and galectin-3 are found as soluble proteins in human serum. Soluble CD23 (sCD23) is a modulator of IgE responses in vivo and is generated by cleavage of membrane CD23 from the surface of B-cells [13]. sCD23 has been demonstrated to enhance IgE production [14,15,16] and several reports show that high serum levels of sCD23 correlate directly with the severity of allergy and asthma [17]. Along this line, successful immune therapy is accompanied by a drop in sCD23 levels in the serum of allergic patients [18]. The role of sCD23 in modulating IgE production and its potential for monitoring allergic responses has been discussed for more than two decades [13,19,20]. However, sCD23 is currently approved as a prognostic parameter only for B-cell chronic lymphocytic leukemia [21,22,23]. Interestingly, soluble galactin-3 is also a common marker for tumor burden [4,24]. Why the production of these soluble IgE receptors is induced during malignant diseases is an interesting scientific question that has yet to be resolved. Thus, our limited understanding of the in vivo role of sCD23 and soluble galectin-3 highlights the need for continued research on soluble factors that modulate serum IgE responses in the context of an allergic response.

FcεRI is an activating immune receptor of the immunoglobulin superfamily, which includes the Fc receptors CD16, CD32, CD64 and CD89 [6,25,26]. FcεRI shares key structural characteristics and signaling features with these Fc receptors. For most IgE, IgG and IgA Fc receptors, soluble isoforms are found in humans. FcεRI, however, has so far not been reported as a soluble IgE receptor in human serum [1,6].

Here we describe a soluble form of the FcεRI alpha-chain (sFcεRI). In human serum, this sFcεRI is found as both a free form and bound to its ligand IgE. We show that IgE-mediated cell activation induces the release of sFcεRI in vivo and that the soluble...
form of the receptor can inhibit binding of IgE to FcεRI at the cell surface.

Results

Detection of a soluble form of FcεRI alpha (sFcεRI) in human serum

To give a definitive answer whether a soluble form of the alpha chain of FcεRI exists in humans, we performed immunoprecipitation experiments to isolate this protein from serum. Sera from patients with normal IgE levels and elevated IgE were run over IgE-columns. Eluates from these columns were analyzed with the patients with normal IgE levels and elevated IgE were run over IgE-columns. Eluates from these columns were analyzed with 12% non-reducing SDS-PAGE gels, transferred to PVDF membranes and probed with anti-FcεRI alpha (sFcεRI) mAb 19-1 followed by peroxidase (HRP)-conjugated goat-anti-mouse IgG for detection of precipitated FcεRI-alpha. The molecular weight is given in kDa.

Detection of sFcεRI in human serum by ELISA

To allow for semi-quantitative analysis of sFcεRI levels in human serum, we next established a sandwich-ELISA system (schematic model in Figure 2A). For this ELISA, the anti-FcεRI-alpha mAb CRA1 was used as the capture antibody. This mAb binds the stalk region of the alpha-chain and is expected to capture a serum alpha-chain without interfering with the IgE-binding epitope [29]. Levels of serum sFcεRI dropped when comparing pre- and post-immunoprecipitation samples (Figure 2B), confirming the specificity of our ELISA. As a further control, capture and detection antibodies were omitted, which consistently resulted in a loss of sFcεRI signal (data not shown). We were also able to detect sFcεRI in plasma with this ELISA, (data not shown). Using a small collective of atopic pediatric patients (5 boys, 3 girls, mean age 10.3+/−2.7 years; detailed patient characteristics are found in Table S1), we established that this ELISA is a feasible method for detection of sFcεRI in a larger patient set. In summary, this set of data describes a novel ELISA for the detection of serum sFcεRI. Any conclusions about the clinical relevance of this finding are however not possible based on the small patient collective.

Serum levels of sFcεRI correlate with serum IgE levels in patients with elevated IgE

Due to the absence of a recombinant sFcεRI protein for the generation of a standard curve, the specific, blanked OD was used for semi-quantitative analysis of serum sFcεRI levels. To investigate the occurrence of sFcεRI and potential associations

Figure 1. A soluble form of the high affinity IgE receptor, FcεRI, is found in human serum. A. Immunoprecipitations from a negative (first lane, Serum 1) and a positive (right lane, Serum 2) serum specimens. Soluble FcεRI (sFcεRI) was precipitated from serum with IgE-loaded NIP-beads and eluted with non-reducing Laemmli sample buffer. Eluates were separated on 12% non-reducing SDS-PAGE gels, transferred to PVDF membranes and probed with anti-FcεRI-alpha mAb 19-1 followed by peroxidase (HRP)-conjugated goat-anti-mouse IgG for detection of precipitated α-chain. B. Comparison of sFcεRI from serum (upper left blot) with FcεRI precipitated from the cell surface of MelJuso-αγ cells (upper right blot). In the low molecular weight range, blots were re-probed with an anti-FcεRI-gamma polyclonal serum. sFcεRI does not associate with the gamma chain (lower panel). Molecular weight is given in kDa.

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with serum IgE in pediatric patients, we screened sera from a cohort of 119 children (56 boys, 63 girls, mean age 10.6+/−5.3 years) with a wide range of normal and elevated serum IgE levels. Patients were categorized based on the specifications given in the Materials and Methods section into individuals with normal or elevated IgE. We found a weak correlation between serum IgE and sFcRI in patients with elevated IgE (n = 32, rho = 0.291, p = 0.106, Spearman’s rank correlation, Figure 2C). In children with elevated IgE-levels, levels of sFcRI and total IgE levels correlate. In children with normal serum IgE-levels, sFcRI could be detected, but no correlation with total IgE levels was found. E. sFcRI circulates as a free or an IgE-complexed protein in human serum. By omitting the IgE-loading step in the ELISA protocol, circulating complexes of IgE and sFcRI were measured. The fraction of free sFcRI was then calculated as OD(total sFcRI)−OD(IgE- sFcRI complexes) = OD (free sFcRI). Graph displays the 14 patients with the highest OD (total sFcRI) with an arbitrary cut off of >0.15.

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IgE-antigen-mediated receptor crosslinking induces the production of sFcRI from an FcRII-expressing cell line

The mechanism of sFcRI production cannot be studied using primary human cells due to limited access to patient material. We thus took advantage of a recently established cell line that allows studying the function of trimeric FcRII in vitro. This new cell line model is based on MeJuso cells, which were stably transfected with FcRII-alpha and FcRII-gamma cDNA. The resulting MeJuso-αγγ cells express FcRII-alpha at the cell surface (Figure 3A) and can bind monomeric IgE (Figure 3B, left FACS histogram). In line with previous reports, IgE binding to MeJuso-αγγ also induces upregulation of surface FcRII-alpha, a key feature of this Fc receptor (Figure 3B, right FACS histogram). Multimeric FcRII complexes containing FcRII-alpha and FcRII-gamma subunits can be precipitated from this cell line (Figure 3C) and receptor activation by crosslinking of FcRII induces efficient receptor internalization from the cell surface (Figure 3D). All of these features match the characteristics of trimeric human FcRII found in the literature [10,11,12]. Thus, we used this cell model to address whether IgE-mediated activation of cell-surface FcRII induces the release of the soluble form of the receptor. MeJuso-αγγ cells were loaded overnight with hapten-specific cIgE. After removal of excess cIgE, surface FcRII was activated by crosslinking the receptor-bound ligand with haptenized antigen. 36 h after receptor crosslinking, sFcRII was precipitated from culture

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Figure 2. Detection of sFcRI in human serum by ELISA. A. Schematic of the ELISA established for the detection of sFcRI. B. ELISA measurements pre- and post-immunoprecipitation with IgE-loaded NIP-beads confirmed that IgE immunoprecipitation depleted serum of sFcRI. OD, optical density at 450 nm. C. In children with elevated IgE-levels, levels of sFcRI and total IgE levels correlate. D. In children with normal serum IgE-levels, sFcRI could be detected, but no correlation with total IgE levels was found. E. sFcRI circulates as a free or an IgE-complexed protein in human serum. By omitting the IgE-loading step in the ELISA protocol, circulating complexes of IgE and sFcRI were measured. The fraction of free sFcRI was then calculated as OD(total sFcRI)−OD(IgE- sFcRI complexes) = OD (free sFcRI). Graph displays the 14 patients with the highest OD (total sFcRI) with an arbitrary cut off of >0.15.

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supernatants with a cIgE column and visualized by immunoblotting with mAb 19-1 and compared to sFc 
  
Figure 3. sFcRI is released from a cell line after IgE-antigen-mediated receptor crosslinking. A. Surface FcεRIα expression of MelJuso-αγ cells (black histogram) compared to MelJuso cells (filled gray histogram) and isotype control (grey hedged histogram). B. Detection of IgE binding of MelJuso-αγ cells with NP-PE (left overlay) and of increased surface FcεRIα expression induced by IgE binding (right overlay). Cells were cultured for 16 h in the presence or absence of cIgE. Black histograms represent cells incubated with cIgE; grey filled histograms are non-cIgE treated cells; isotype control is shown as gray, hedged histogram. C. Coimmunoprecipitation of FcεRI alpha and gamma-chain dimers from MelJuso-αγ cells. D. FcεRI internalization induced by antibody-mediated crosslinking. FcεRI is shown in red and cell surface membranes were stained with WGA (in green). Representative images of cells with non-crosslinked FcεRI (t = 0; left image) and cells 45 min after crosslinking of FcεRI (t = 45 min; right image). E. After 36 h of crosslinking, sFceRI can be precipitated from supernatants of activated MelJuso-αγ; sFceRI proteins were precipitated with IgE-loaded beads, eluted with non-reducing Laemmli sample buffer, separated on 12% non-reducing SDS-PAGE gels, transferred to PVDF membranes, and probed with anti-FcεRI-alpha (mAb 19-1) followed by peroxidase (HRP)-conjugated goat-anti-mouse IgG. F. Kinetics of sFceRI release into culture supernatants. Supernatants were harvested from MelJuso-αγ cell cultures 4, 8, 24 and 32 h after receptor activation (left graph). ELISA measurements showed an accumulation of sFceRI over time. MelJusoØØ does not produce sFceRI (right graph). G. sFceRI is a soluble protein as it could be detected in culture supernatants (left lane) and in exosome-depleted culture supernatants (right lane). SN: supernatant; OD: optical density. Molecular weight is given in kDa.

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supernatants with a cIgE column and visualized by immunoblotting with mAb 19-1 and compared to sFceRI precipitated from patient serum (Figure 3E). Next, the kinetics of sFceRI release was studied by harvesting supernatants 4, 8, 24 and 32 h after receptor crosslinking for analysis by ELISA. Accumulation of sFceRI was observed only after receptor crosslinking (Figure 3F, left graph). sFceRI was not detected in supernatants of empty vector-transfected MelJusoØØ cells that do not express FcεRI (Figure 3F, right graph). To demonstrate that the detected protein was a soluble form of FcεRI and not protein shedded with exosomes or derived from cell debris, sequential high-speed ultracentrifugation was performed to deplete the supernatants from cell debris and exosomes as established by Thery et al. [30]. sFceRI was detected in the exosome-depleted, soluble fraction after high-speed centrifugation confirming that the detected protein is a bona fide soluble form of the receptor (Figure 3G).

sFceRI inhibits IgE loading of FcεRI at the cell surface in vitro

Since we detected IgE-sFceRI complexes in serum, we speculated that sFceRI could interfere with IgE-binding to FcεRI
when expressed at the cell surface. If that indeed occurs, sFcεRI could function as a potential modulator of IgE-mediated immune activation. We tested this hypothesis by loading FcεRI-expressing MelJuso-εγ cells with either a mix of clgE and cell-culture derived sFcεRI or with clgE diluted with medium control. Cell-bound clgE was visualized by flow cytometry with PE-conjugated hapten NP. sFcεRI efficiently blocked binding of clgE to FcεRI expressed at the cell surface (Figure 4A). Binding of clgE was blocked in a dose dependent manner as dilution curves with supernatants from sFcεRI-containing MelJuso-εγ cells and control supernatants from inactivated cells demonstrated (Figure 4B). In summary, these results show that sFcεRI can interfere with binding of IgE to FcεRI at the cell surface of immune cells.

**Discussion**

We here describe a soluble version of the FcεRI-alpha (sFcεRI) chain that circulates in human serum as a free protein or bound to its natural ligand, IgE. We show that sFcεRI is released upon IgE-antigen-mediated activation of cell surface FcεRI in vitro and, maybe most interestingly, that sFcεRI interferes with IgE-binding to cellular FcεRI in vitro. The affinity of IgE with its high affinity receptor FcεRI was defined after crystallization of the ligand with recombinant version of the alpha chain [31,32]. It is therefore highly likely that the soluble form in human serum has equally high affinity as described in the literature.

Commonly, the reagents used to detect transmembrane forms of FcεRI-alpha are directed against the IgE-binding epitope of the protein. Thus, the identification of sFcεRI could easily have been missed if the detection reagents were not selected carefully. We here established an ELISA system that uses a monoclonal antibody directed against the stalk region of the protein [29] to capture sFcεRI and use human IgE combined with anti-IgE for detection [12]. By omitting the IgE incubation step, this ELISA also allows for an assessment of the amount of sFcεRI that circulates as a preformed complex with serum IgE.

Several studies with recombinant versions of sFcεRI are found in the literature [33,34]. Since the recombinant sFcεRI used as a tool to interfere with allergic responses and a potential therapeutic agent, there have been some speculations about a soluble serum equivalent in the literature [8]. A single report is found in the literature that described a soluble complex of FcεRI in cultures of human eosinophils [35]. Since the integrity of FcεRI complexes requires the presence of cell membranes [28,36], Seminario et al. most likely described a version of the receptor that was released in an exosomal fraction rather than a bona fide soluble protein.

Based on our current understanding of the mechanism of sFcεRI generation, it is fair to assume that serum sFcεRI is a reflection of FcεRI activation. In an independent study, we were able to confirm the observation of Liang et al. [37] showing that patients can carry substantial amounts of IgE on peripheral blood cells even in the absence of elevated serum IgE [27]. In summary, these two studies show that cells in the peripheral blood bind IgE from the serum and thereby can clear the serum of IgE. These IgE-loaded cells could be the source of sFcεRI when activated.
Our finding that the presence of sFceRI correlates with serum IgE supports this hypothesis. On the other hand, IgE-mediated cell activation could also account for the detection of serum sFceRI in the absence of high serum IgE levels. Whether patients that have high sFceRI are protected from allergic diseases will have to be addressed in detail. Along this line of argument, it is tempting to speculate that serum sFceRI is a predictive marker for the onset of allergies that may be detectable even before serum IgE levels are elevated. We are currently investigating this hypothesis in a prospective cohort study.

sFceRI is also an excellent candidate for an efficient in vivo modulator of IgE-mediated responses. While sCD23 has to trimerize to develop considerable affinity for its ligand [1], sFceRI can bind IgE with a one-to-one ligand-receptor ratio. Additionally, the affinity of the FceRI-IgE interaction is exceptionally high and disruption of a once formed contact requires low pH, which is physiologically found only in the stomach [1,6,7]. The finding that receptor crosslinking is required for the production of sFceRI also hints at a potential negative feedback mechanism. Antigen-IgE-mediated receptor crosslinking could induce shedding of sFceRI to remove IgE-binding sites from the cell surface and to terminate receptor-mediated signaling. In addition, we show here that sFceRI has the ability to prevent IgE-binding to surface expressed receptors. Thus the presence of serum sFceRI could inhibit IgE-loading of effector cells of allergy in vivo.

Our in vitro studies suggest that sFceRI may also prevent IgE-mediated activation of the immune system by clearing the serum of IgE in a manner comparable to omalizumab. Omalizumab is a recombinant humanized monoclonal antibody directed against serum IgE and currently approved for the treatment of severe allergic asthma [38,39,40]. Omalizumab also downregulates cell surface levels of FcεRI [41].

In summary we here describe a new soluble form of human FcεRI, the high affinity receptor for IgE (s FceRI), in human serum. Establishing an improved quantitative ELISA with a standard protein is now highly important, because such a quantitative method will allow for an extensive comparative analysis of serum levels in various patient groups. Such studies will be of outmost importance to draw conclusions about the clinical relevance of this new serum IgE receptor. It will be also essential to gain a better understanding of how endogenous levels of sFceRI are regulated and how sFceRI is linked to IgE-mediated immune activation in vivo.

Materials and Methods

Ethics statement

Patient sera used for this study were obtained from an ongoing prospective cohort study on the role of FcεRI in the gastrointestinal tract at Children’s Hospital Boston or had been previously obtained as part of routine clinical care at the Medical University of Vienna. The prospective cohort study was approved by the Investigational Review Board of Children’s Hospital Boston (Harvard Medical School, Boston, MA) and patients or their legal guardians provided written informed consent. The retrospective study of patient sera was approved by the Ethics Committee of Medical University of Vienna, Vienna, Austria.

Antibodies and reagents

Anti-human FcεRI alpha mAb 19-1 was kindly provided by Dr. J.P. Kinet (Laboratory of Allergy and Immunology, Beth Israel Deaconess Medical Center, Boston, MA) and used as previously described [12,28,36]. Anti-human FcεRI alpha mAb CRA1 (clone AER-37) was purchased from eBioscience, San Diego, CA. Anti-FcεRI-gamma polyclonal serum was purchased from Millipore, Billerica, MA. Chimeric IgE that contains the human Fc domain and recognizes the haptens 4-hydroxy-3-nitrophenylacetic acid (NP) and 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) with its Fab region (cIgE) was derived from Jw 8/5/13 cells (Serotec, Oxford, UK), kindly provided by Dr. D. Maurer, Department of Dermatology, Medical University of Vienna, Austria, [12,42] and was used for immunoprecipitation of properly folded FcεRI-alpha and for in vitro cell culture experiments. Phycocerythrin (PE)-conjugated was purchased from Biosearch Technologies, Novato, CA, and used for flow cytometry analysis. Anti-mouse IgG (Fc specific, produced in goat; Sigma Aldrich, St. Louis, MO, #M3534-1 mL) was used for coating of the ELISA plates. High-IgE human serum (total IgE=2000 kU/L) was purchased from Bioreclamation, Hicksville, NY to assure the quality control of IgE used for detection of sFceRI by ELISA. Goat anti-human IgE HRP conjugated antibody (CalTag, Invitrogen, Carlsbad, CA) was used as a secondary antibody.

Cell lines and culture conditions

The MelJuso cell line was provided by Hidde L. Ploegh (Whitehead Institute, MIT, Cambridge, MA) and is well-established for studies on MHC class I and MHC class II trafficking [43]. MelJuso cells that stably express FcεRI-alpha and FcεRI-gamma (MelJuso-γγ) were generated by viral transduction using a standard protocol (http://www.stanford.edu/group/nolan/) following the guidelines of Children’s Hospital Boston. MelJuso cells transfected with empty vector (MelJusoØØ) and MelJuso-γγ cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Cellgro, MediaTech, Herndon, VA) supplemented with 10% fetal calf serum (HyClone, Logan, UT), 2 mM glutamine (Cellgro), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco BRL, Gaithersburg, MD). Cells were resuspended using hygromycin (1 mg/ml) and puromycin (1 µg/ml, both Sigma). Jw 8/5/13 cells [12,42] were used for the production of cIgE and cultured in RPMI 1640 medium (Gibco, Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (HyClone), 2 mM glutamine (Cellgro), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco BRL).

Patient sera

Sera from adult individuals were tested for the presence of sFceRI by immunoblot and ELISA. Sera from eight polysensitized, highly atopic children were analyzed for sFceRI by ELISA. Total serum IgE and allergen-specific IgE were measured by solid phase immunoassay (Phadia ImmunoCAP®, Pharmacia Diagnostics, Uppsala, Sweden). Total serum IgE levels are given in kU/L, specific IgE is given in kUA/l and CAP RAST classes.

Sera from 119 children were obtained from an ongoing prospective cohort study on the role of FcεRI in the gastrointestinal tract. Patients between 1 and 19 years of age scheduled for an elective esophago-gastro-duodenoscopy at the Division of Gastroenterology at Children’s Hospital Boston were randomly invited to participate. Subjects who used steroids in any form, immunomodulatory drugs, mast cell stabilizer, or leukotriene inhibitor within the last 3 months, as well as patients with an established diagnosis of autoimmune, inflammatory, or immunodeficiency disease were not enrolled. Total serum IgE levels were assessed according to standard procedures at Children’s Hospital Boston using the Elecsys IgE II kit (Roche Diagnostics, Mannheim, Germany). IgE levels are given in kU/L. Expected normal ranges for this assay are 30 kU/L for age 0–3 years, 200 kU/L for 3–10 years, 500 kU/L for 10–14 years, and 200 kU/L for >14 years.
Immunoprecipitation and immunoblotting of sFceRI

To target the fully mature form of FceRI alpha as expressed on the cell surface, we loaded MelJuso-γγ cells with cIgE (10 mg/ml in PBS) before solubilization in lysis buffer (3×10^6 cells per ml; 0.5% Brij 96, 20 mM Tris, pH 8.2, 20 mM NaCl, 2 mM EDTA, 0.1% NaN₃) containing protease inhibitors (Complete, Roche, Genentech, South San Francisco, CA) for 30 min on ice. Immunoprecipitation was next performed with NIP-heads (Sigma) as previously described [36,42]. Proteins were eluted from beads in non-reducing Laemmli sample buffer and samples were separated on 12% non-reducing SDS-PAGE gels, transferred to PVDF membrane (Pierce, Thermo Fisher Scientific, Rockford, IL) and probed with anti-FceRI-alpha (mAb 19-1 or CRA1 for reducing conditions, both 0.5 mg/ml) followed by peroxidase (HRP)-conjugated goat-anti-mouse IgG for detection of precipitated α-chain. For immunoprecipitation of sFceRI from serum, 2–5 ml serum was used. cIgE-loaded NIP columns were also used for purification of sFceRI from supernatants of MelJuso-γγ cells prior to immunoblotting. Peroxidase activity was detected by SuperSignal chemiluminescent substrate (Pierce). Accordingly, sFceRI was precipitated from serum with IgE-coupled beads and immunoblotting was performed.

For co-immunoprecipitation of FceRI alpha and gamma chains from MelJuso-γγ cells, cells were loaded overnight with cIgE. Cell lysates were prepared and incubated with NIP sepharose beads as described above. FceRI alpha-chain was detected with mAb 19-1, FceRI gamma-chains with polyclonal rabbit anti-FceRIγ antibodies (Upstate).

ELISA for the detection of sFceRI in cell culture supernatants and serum

To improve sensitivity, wells were first incubated with a goat-anti-mouse coating antibody (5 mg/ml, Sigma), then with anti-alpha chain mAb (CRA1 0.5 mg/ml, clone AER-37; eBioscience). After a blocking step with 10% FCS in PBS, wells were incubated with sera (1:2 dilution) overnight. After repetitive washing, plate-bound sFceRI was loaded with IgE (Bioreclamation) and detected with a goat anti-human IgE-HRP conjugated second step (Caltag). Rather than using IgE purified from different patient sera, IgE was used to ensure the quality and consistency of this reagent in our ELISA. Conversion of 3,3′,5,5′-tetramethyl-benzidine liquid substrate (TMB, Sigma) was measured at 450 nm. Results are given as optical density (OD). To control for intra-assay variability, we included on each plate a positive and a negative control as previously described [36,42]. Levels of free sFceRI were therefore calculated as follows: OD_{total}−OD_{IgE}−sFceRI_{complexes} = OD_{free}−sFceRI.

In a subset of patients, sFceRI was measured in plasma and serum in parallel. For conversion of plasma samples into serum, BD Serum Separation Tubes (Becton Dickinson) were used according to the manufacturer’s guidelines.

Production of sFceRI by MelJuso-γγ cells and detection in cell culture supernatants

MelJuso-γγ cells were grown to confluence and incubated with cIgE overnight. Excess cIgE was washed away and ligand-bound receptor was activated with haptenized antigen (BSA- or OVA-, 1 μg/ml, both from Biosearch Technologies, Novato, CA). Cell culture supernatants were collected after the indicated time periods and analyzed for the presence of soluble alpha chain by ELISA or by immunoprecipitation.

Exosome removal

To remove exosomes from cell culture supernatants, MelJuso-γγ supernatants were treated with a sequence of ultracentrifugation steps following the protocol published by Thyry et al. [30]. Briefly, exosome-free supernatants were obtained by the following consecutive centrifugations: 300 g for 3 minutes, 1200 g for 20 min, and 10000 g for 30 min, followed by a final centrifugation step at 110000 g for 1 h.

Flow cytometry analysis

Surface expression of FcεRI on MelJuso-γγ cells was determined by staining with the anti-human FcεRI alpha mAb CRA1. IgE binding was tested by culturing MelJuso-γγ cells in the presence or absence of NP-specific cIgE and staining with phycoerythrin (PE)-conjugated NP (NP-PE; Biosearch Technologies). For the detection of sFctRI, cells were loaded with either a mix of cIgE (100 ng/ml) in culture supernatants that contained sFceRI or supernatants from unstimulated cell cultures for 30 min on ice. A number of different ratios of cIgE to culture supernatants was analyzed. FcεRI-bound cIgE was stained with NP-PE. Analysis was performed on a BD FACScan™ flow cytometer using CellQuest software for acquisition and data analysis (both from Becton Dickinson).

Immunofluorescence Microscopy

For FcεRI alpha internalization, MelJuso-γγ cells were grown on coverslips (No 1.5), stained first with purified mouse anti-FcεRI alpha CRA1 antibody for 20 min at 37°C and subsequently with an anti-mouse Alexa Fluor 568 for 45 min at 37°C to induce receptor crosslinking. Cells were fixed with 4% paraformaldehyde for 20 min and mounted using Prolong Antifade reagent (Invitrogen). Both antibodies were diluted in HBSS supplemented with 10 mM HEPES (Invitrogen) and 5% NuSerum (Invitrogen). CRA1 was diluted at 1:100 and anti-mouse Alexa Fluor 568 was diluted at 1:400. Plasma membranes of fixed cells were stained with Alexa Fluor 647-conjugated wheat germ agglutinin (WGA, diluted at 1:1000) for 10 min. For time point t = 0 cells were fixed before incubation with anti-mouse Alexa Fluor 568 and WGA. All incubation steps were carried out in a humidified chamber.

Confocal images were acquired on a Nikon TE2000 inverted microscope coupled to a Yokogawa spinning disk confocal unit (Perkin-Elmer Inc.) and an Orca AG scientific-grade cooled CCD camera (Hamamatsu Photonics K.K.). Slidebook software (Intelligent Imaging Innovations Inc.) was used for image capture, processing, and analysis.

Statistical Analysis

Correlations between serum IgE and serum sFceRI were calculated by Spearman’s rank correlation test using SPSS for Windows (version 16.0, SPSS Inc., Chicago, IL). Spearman’s rank correlation coefficients are displayed as ‘rho’, a p-value of >0.05 was considered significant.

Supporting Information

Table S1 Serum levels of sFceRI in atopic patients. (DOC)

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