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Human serum substitution by artificial sera of scalable allergen reactivity based on polyclonal antibodies and chimeras of human FcγRI and IgE domains

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Short title: Establishment of Artificial Sera

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Key words: allergen specific antibodies; artificial sera; FcγRI (CD64); immunoglobulin E (IgE); standardization material

Abstract:

Human sera are the first choice as controls for diagnostic applications like immunoassays, but are limited regarding availability, varying quality and high costs. In this study we aimed to circumvent these limitations by the use of a chimeric adaptor molecule comprising the extracellular domains of the human FcγRI (CD64) fused to human IgE Fc domains (CD64-IgE Fc). Allergen-specific antibodies were produced in rabbits using 8 different allergens, extracts and allergen mixtures including mites, pollen, drugs, food and others. Pre-incubation of polyclonal IgG with CD64-IgE Fc established allergen-specific artificial sera that showed comparable results for more than 20 allergens and allergen extracts in three diagnostic systems for determination of specific IgE. The agreement for these artificial sera is within ± 1 RAST class. Hence, rabbit IgG complexed with the IgG-specific CD64-IgE Fc adaptor molecule could provide a substitute for human reference sera with specificity for virtually any protein of interest.

Background:

Until now no standardized reference controls for the determination of allergen-specific immunoglobulin (IgE) are available. Nevertheless, for the development and validation of allergy *in vitro* and *in vivo* diagnostics, the use of well-defined quality controls is required. The *in vitro* diagnostic guideline (IVD 98/79/EG) suggests to perform tests of patient samples only in tandem with consistent references. Such human control sera are strongly limited regarding availability, varying quality and high costs. The availability of these controls became increasingly challenging with the increased number of available allergens. In

addition, larger quantities of well characterized sera are required due to the growing number of allergy test performances (1).

Another challenge is the fact that allergen extracts in allergen-specific IgE assays may differ because of variations in the allergen source material, manufacturing process, storage conditions and acceptance criteria (2). To ensure the consistency of allergen extracts, the manufacturers of *in vitro* diagnostics may hold control serum samples with the specificity to any allergen available. However, the reproducibility within a single manufacturer's extract production cannot guarantee a good accordance between test results of assay systems from different manufacturers (3). Comparison of allergen-specific IgE assays can be performed by the use of round robin test material. However, the scarcity of human sera with sufficient levels of sIgE to even less prominent allergens of interest and the strong limitations regarding quantity of available sera also hamper adjustment of different assay systems (4).

Approaches to address these needs for allergen-specific sera are not in reach. The use of recombinant monoclonal IgE allows to address very specific diagnostic questions (5) but is hampered by the high effort and the narrow specificity. Moreover monoclonal IgE cannot mimic the broad epitope distribution given in a human serum. Therefore the aim of this study was to circumvent these limitations by establishing a versatile tool which allows the use of polyclonal antibodies with specificity for any allergen of interest in immunoassays.

Methods:

The extracellular domains (ECD) of the human high affinity IgG receptor FcγRI (CD64) (6) were fused to human immunoglobulin Fc regions of the epsilon isotype (IgE) (7, 8). The recombinant adaptor molecule comprising the FcγRI ECD and IgE Fc domains (CD64-IgE Fc) was expressed in human HEK-293 cells (**Fig. E1, online repository**). Purification was

based on a two-step procedure using Ni-affinity chromatography followed by size exclusion chromatography. Binding of CD64-IgE Fc to antibodies from different species was assessed in ELISA as described in the **online repository**.

Allergen-specific IgG antibodies were produced in rabbits by immunization with purified recombinant and native allergens as well as whole allergen extracts. Pre-incubation of polyclonal rabbit IgG with CD64-IgE Fc produced artificial allergen-specific reagents, which were used in different types of allergen-specific immunoassays. A set of such artificial sera was tested in three established assay methods: ALLERG-O-LIQ, ALFA (Dr. Fooke Laboratories) and ImmunoCAP (Thermo Fisher Scientific).

The ALLERG-O-LIQ is based on the “reversed enzyme allerge sorbent test (REAST)” by using anti-human IgE coated microtiter plates in combination with biotinylated reagents (9). In contrast, in the ImmunoCAP system (direct immunoassay) the allergens are bound to a solid phase (10). ALFA “allergy lateral flow assay” is a rapid test consisting of a universal test cassette with variable allergens which were biotinylated and used as liquid sources (11).

More than 20 artificial serum samples with different allergen specificities were tested in all three immunoassays. The concentration (kU/L for ImmunoCAP, IU/mL for ALLERG-O-LIQ and U/mL for ALFA) and the corresponding class were calculated for each artificial serum sample tested in the mentioned assays. For titration experiments, the artificial allergen-specific reagents were diluted in two-fold steps until the measured concentration was lower than 0.35 IU/mL. These serially diluted sera were analyzed in all immunoassays.

Results and discussion:

The CD64-IgE Fc adaptor molecule was efficiently produced and secreted into the supernatant of HEK-293 cells and could be purified by Ni-NTA affinity chromatography under mild elution conditions followed by size exclusion chromatography. PAGE and immunoblot analyses suggested proper assembly into the homodimeric format and glycosylation of the CD64 and IgE Fc domains (**Fig. 1A**). Notably, the yields of the CD64-IgE Fc fusion protein are significantly higher than those of soluble CD64 alone (12).

The purified CD64-IgE Fc adaptor molecule was used for analyses of its reactivity and specificity in ELISA. The CD64-IgE Fc adaptor molecule exhibited pronounced immunoreactivity to different IgG antibodies (**Fig. 1B**) confirming the knowledge about its specificity for human IgG subclasses IgG1, IgG3 and IgG4 (13). Similarly human CD64 binds to murine IgG2a, IgG2b and IgG3 but not to IgG1 (14), a finding that is in accordance with the lack of interaction of murine Fc γ RI with IgG1 (15). The immunoreactivity of CD64 to IgG from other species revealed a heterogeneous picture with a pronounced binding to rabbit, sheep and donkey IgG. These immunoreactivities appear to be similar to that of human IgG, suggesting a comparable high affinity binding. Dissociation constants of CD64 and human IgG have been reported to be in the nano- to pico-molar range (12-14). Human IgM, IgA and IgE, goat and bovine IgG, and avian IgY exhibited no immunoreactivity at all. The lack of reactivity to bovine IgG is advantageous when using medium containing fetal calf serum for production.

The specificity of human CD64 allows for using a variety of animals as source for immune sera. Rabbits represent the most commonly used animal for immunization purposes and a variety of polyclonal and monoclonal antibodies are commercially available. Hence we used

the CD64-IgE Fc and rabbit-derived IgG antibodies obtained by immunization to establish artificial sera of defined specificity for selected allergens representing pollen, food, mold, latex and drug allergies. These artificial sera showed comparable results in three established diagnostic systems for the determination of specific IgE (**Tab. 1**). All samples were adjusted to RAST class 2 to RAST class 4 (0.7 IU/mL – 50 IU/mL). The best agreement was given for the artificial serum samples specific for the native purified allergens α -lactalbumin (f76), followed by β -lactoglobulin (f77), penicilloyl-G (c1) and the major allergen from birch Bet v 1. Artificial sera specific for allergen extracts from birch (t3), *Aspergillus fumigatus* (m3), *Dermatophagoides pteronyssinus* (d1), *Dermatophagoides farinae* (d2), hazelnut (f17) and *Hevea brasiliensis* (k82) showed higher deviations, probably due to the complexity of the source material as compared to purified allergens. The agreement however for each of more than 20 artificial sera, specific for recombinant or native allergens as well as for allergen extracts, is within ± 1 RAST class (**Tab. 1**).

Correlation of titrated samples showed Pearson's correlation coefficients (Pearson's r) > 0.99 between ALLERG-O-LIQ vs. ImmunoCAP and ALFA vs. ImmunoCAP for d1, f17 and Bet v 1 (**Fig. 2C-E**). In spite of the excellent correlation for all three allergens, the results for the recombinant Bet v 1 showed a better accordance than those for the allergen extracts. The deviations between the results seem to be independent on the test system, because all three systems exhibited excellent accordance with the recombinant and native purified allergens.

The better agreements for the single allergens compared to the allergen extracts could rely on differences in manufacturer's extract production and varying immune response upon immunization. In order to address this question, rabbits were immunized with a mixture of purified allergens and extract and resulting artificial sera were tested on extract and single

allergens (**Fig. 2F, G**). After immunization with a mixture of recombinant latex allergens (Hev b 5, Hev b 6, Hev b 7) resulting artificial sera showed pronounced reactivity to the single allergens tested with ALLERG-O-LIQ and ALFA and to the whole latex extract in all applied diagnostic systems. The artificial sera produced by immunization with a hazelnut extract showed positive signals to the available allergen components (Cor a 1, Cor a 8, and Cor a 11) tested with ALLERG-O-LIQ and ALFA. Independent of the material for immunization, a better agreement between all three immunoassays is given for the allergen components than for the allergen extracts.

A clear advantage of our approach is that the IgG pool of immunized animals provides a virtually unlimited access to allergen-specific antibodies needed for artificial sera. Although the IgE repertoire of human sera might recognize specific and eventually restricted epitopes, the significantly higher clonality of the IgG repertoire is most likely sufficient to include all IgE epitopes. Moreover, the general flexibility of immunization procedures allows for adjusting specificity, affinity, size and number of epitopes, etc. to the particular needs. Against this background there is no need for human IgE repertoires for standardization purposes. Together with the efficient production of the CD64-IgE Fc adaptor molecule a robust concept for establishing highly specific tools of tailored characteristics in large amounts is given.

Conclusion:

Artificial sera have the potential to provide a substitute for human reference sera with specificity for virtually any target structure of interest including proteins, peptide epitopes, carbohydrates, and drugs. Such artificial sera can be considered as useful tools for the quality control of allergens and allergen extracts and allow for improving standardization,

minimizing lot to lot variations and overcoming limitations of current assay standardization protocols.

Author contributions

The study was conceived by NO, MP, MF, and ES. NO and MP performed the experiments. KR contributed to the ELISA experiments. NO, MP, MF and ES wrote the manuscript. All authors contributed to the interpretation of the data and critical review of the manuscript.

Conflict of interest

NO, UH, MF are employees of Dr. Fooke Laboratories, manufacturer of assay systems used in the study. ES has received research support from Dr.Fooke Laboratories and is cofounder of PLS Design GmbH. The other authors have no financial, research, organizational, or other interests to disclose that are relevant to the execution of this research or this publication.

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Table 1: Establishment of a panel of artificial sera

Artificial sera with IgE reactivity to a variety of allergens and allergen extracts were assembled the CD64-IgE Fc adaptor molecule and allergen-specific IgG antibodies and evaluated in three established diagnostic systems. Allergen-specific IgE concentrations (kU/L for ImmunoCAP, IU/mL for ALLERG-O-LIQ and U/mL for ALFA) and the corresponding class were calculated for each artificial serum sample. d1: *Dermatophagoides pteronyssinus*, house dust mite; d2: *Dermatophagoides farinae*, house dust mite; t3: *Betula verrucosa*, birch; f17: *Corylus avellana*, hazelnut; f76 and f77: *Bos domesticus*, cow's milk; k82: *Hevea brasiliensis*, latex; m3: *Aspergillus fumigatus*, mold; c1: Penicilloyl G, antibiotic drug.

Allergen source	Allergens for immunization	Allergens for sIgE detection	Diagnostic system					
			ImmunoCAP		ALLERG-O-LIQ		ALFA	
			KU/L	Class	IU/mL	Class	U/mL	Class
Mites	d1	d1	13,30	3	33,31	4	18,48	4
			3,61	3	14,61	3	6,78	3
			4,39	3	17,25	3	4,7	3
			3,16	2	7,4	3	8,98	3
	d2	d2	12,50	3	12,26	3	6,33	3
			3,00	2	6,12	3	2,68	2
			3,73	3	4,82	3	1,75	2
			3,00	2	10,58	3	2,15	2
Trees	Bet v 1	Bet v 1	7,70	3	4,31	3	9,37	3
			3,10	2	1,09	2	1,76	2
		t3	8,86	3	4,05	3	6,86	3
			3,98	3	1,43	2	1,57	2
Food	f17	f17*	8,22	3	21,96	4	6,52	3
			3,77	2	8,02	3	1,57	2
	f76	f76	4,07	3	6,36	3	5,78	3
			1,88	2	1,16	2	2,28	2
	f77	f77	16,80	3	12,89	3	19,31	4
			7,57	3	4,66	3	8,74	3
Occupation	Hev b 5, 6, 7	k82**	23,30	4	10,03	3	16,37	3
			6,93	3	3,42	2	2,38	2
Molds	m3	m3	2,74	2	8,69	3	4,12	3
			0,83	2	3,2	2	1,16	2
Drugs	c1	c1	11,40	3	16,84	3	13,97	3
			4,84	3	5,47	3	3,49	2

*Data for sIgE detection for f17, Cor a 1, Cor a 8, and Cor a 11 are shown in detail in Fig. 1F

**Data for sIgE detection for k82, Hev b 5, Hev b 6, and Hev b 7 are shown in detail in Fig. 1G

Figure legend:

Figure 1: Generation and specificity of the CD64 IgE Fc adaptor molecule and evaluation of different artificial sera

A: The purified CD64-IgE Fc adaptor molecule was analyzed using PAGE under native and non-reducing (nr) and denaturing and reducing (r) conditions by Coomassie staining.

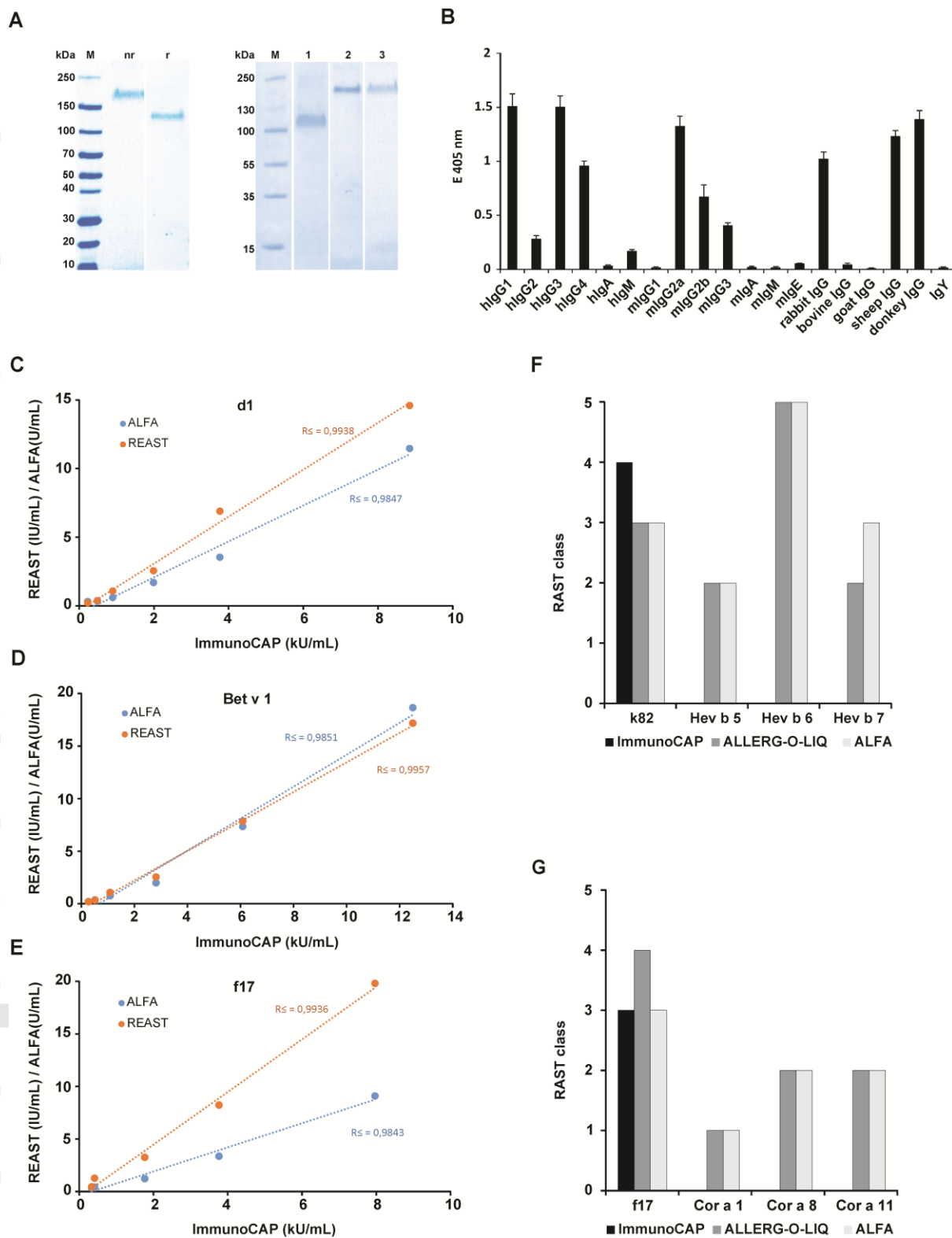
The purified CD64-IgE Fc adaptor molecule was further analyzed by immunoblotting after SDS-PAGE (1) and immunoblotting after a native and non-reducing PAGE using an anti-human-IgE antibody (2). Additionally, a rabbit IgG antibody (3) was used for detection of the CD64-IgE Fc adaptor molecule after native PAGE. The secondary antibodies are both conjugated to horseradish peroxidase and signals were obtained by addition of TMB.

B: Immunoreactivity of CD64-IgE Fc was assessed in ELISA using antibodies of different species and varying isotypes and subclasses that were coated to an ELISA plate. CD64-IgE Fc binding to the coated antibodies was visualized by an AP-conjugated anti-IgE antibody produced in goat. The ELISA was performed in triplicates.

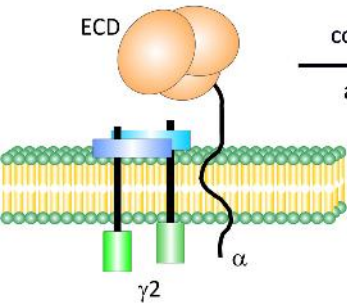
C – E: Correlation studies were performed by the titration of artificial sera specific to d1, f17 and Bet v 1 and tested in three established diagnostic systems (ImmunoCAP, ALLERG-O-LIQ (REAST), and ALFA). Correlation coefficients were calculated by Pearson correlation between the results from ALLERG-O-LIQ and ALFA vs. ImmunoCAP. Correlation coefficients of 0.99 and 0.98 for d1, 0.99 and 0.98 for f17, 0.99 and 1.00 for Bet v 1 were determined between ALLERG-O-LIQ vs. ImmunoCAP and ALFA vs. ImmunoCAP, respectively. d1: *Dermatophagoides pteronyssinus*, house dust mite; f17: *Corylus avellana*, hazelnut.

F: Artificial sera specific to latex (k82) were produced by immunization of rabbits with a mixture of recombinant latex allergens (Hev b 5, Hev b 6, Hev b 7). Resulting allergen-specific rabbit IgG antibodies were coupled to the CD64-IgE Fc adaptor, forming an artificial serum with significant IgE reactions to all recombinant latex allergens (tested with ALLERG-O-LIQ and ALFA) and to the whole latex extract in all applied diagnostic systems (ImmunoCAP, ALLERG-O-LIQ, and ALFA). k82: *Hevea brasiliensis*, latex.

G: Artificial sera specific to hazelnut were produced by immunization of rabbits with f17. Resulting allergen-specific rabbit IgG antibodies were complexed with the CD64-IgE Fc adaptor molecule, forming an artificial serum with significant IgE reactivities to the whole hazelnut extract (tested with ImmunoCAP, ALLERG-O-LIQ, and ALFA) as well as to the recombinant hazelnut allergens Cor a 1, Cor a 8, and Cor a 11 (tested with ALLERG-O-LIQ and ALFA).

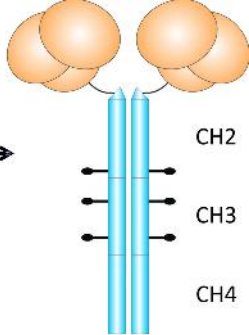


Fc γ RI (CD64)



conversion into

antibody-like
format



CD64-IgE

Supplementary data online repository

Human serum substitution by artificial sera of scalable allergen reactivity based on polyclonal antibodies and chimeras of human FcγRI and IgE domains

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Short title: Establishment of Artificial Sera

Methods:

ELISA

The binding of CD64-IgE Fc to antibodies of different species and varying isotypes and subclasses was assessed in ELISA essentially according to established protocols. Primary antibodies were coated each with a concentration of 100 µg/mL on a 384 well microtiter plate (Greiner). After washing and blocking, purified CD64-IgE Fc diluted at a final concentration of 2 µg/mL in 20 mg/mL milk powder in PBS was incubated for 1 hour at RT. After washing AP-conjugated goat anti-IgE antibody was used for detection in a final dilution of 1:20000 in 20 mg/mL milk powder in PBS. Signals were obtained by addition of substrate solution (5 mg/mL 4-nitro-phenylphosphate, AppliChem, in 100 mM Tris, 10 mM MgCl₂ and 100 mM NaCl, pH 9.5). The plate was read at 405 nm (Infinite® 200 Pro, Tecan).

PAGE, Coomassie staining and immunoblotting

The purified CD64-IgE Fc was analyzed by SDS-PAGE, native PAGE and immunoblotting. SDS-PAGE and native PAGE were performed with precast gradient polyacrylamide gels (4-20%, Mini-PROTEAN TGX™, bio-rad). For SDS-PAGE a running buffer with SDS (25 mM Tris, 192 mM glycine, 0.1% SDS) and Laemmli sample buffer (2x, 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% (v/v) glycerol, 0.01% bromophenol blue) were used. For native PAGE a running buffer without SDS (25 mM Tris, 192 mM glycine) and a sample buffer with native and non-reducing conditions (62.5 mM Tris-HCl, pH 6.8, 40% (v/v) glycerol, 0.01% bromophenol blue) were used. As reducing agent 5% β-mercaptoethanol was added to the sample buffer. Proteins were visualized by Coomassie staining.

Western Blots were done with Trans-Blot® Turbo™ Transfer System (bio-rad). After blocking with 5% (w/v) BSA in TBST, membranes were incubated for 1 hour at RT with anti-human-IgE antibody or rabbit IgG antibody. Both antibodies are conjugated to horseradish peroxidase and were used for detection in a final dilution of 1:5000 in 5% (w/v) BSA in

TBST. Signals were obtained by addition of a precipitating TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution (SeramunBlau[®] prec, Seramun). Size standards from Thermo Scientific were used for PAGE and immunoblotting (PAGE Ruler unstained and prestained).

Figure legends

Figure E1: Schematic representation of the CD64-IgE Fc adaptor molecule

The extracellular domains of the human FcγRI (CD64) α-chain were fused with the Fc-domains CH2-4 of the human IgE heavy chain. The resulting homodimeric adaptor molecule provides an IgG-binding moiety and an IgE Fc that can be used for IgE-specific detection.