SUPPORTING INFORMATION

Immune complexes exposed on mast cell-derived nanovesicles amplify allergic inflammation

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Abbreviations used: Ag: Antigen; DC: Dendritic Cell; DLS: Dynamic Light Scattering; DNP-HSA: DiNitroPhenyl-Human Serum Albumin; ESCRT: Endosomal Sorting Complex Required for Transport; EXO-NT: EXOsome released from uNTreated cells; EXO-T: EXOsomes released from Treated cells; EV: Extracellular vesicle; FcεRI: high affinity Fc Receptor for IgE; Hrs, Hepatocyte growth factor-Regulated tyrosine kinase Substrate; IL: InterLeukin; mBMMC: Mouse bone marrow-derived Mast Cells; MC: Mast Cells; MVB: MultiVesicular Bodies; TEM: Transmission Electron Microscopy.

MATERIAL AND METHODS

Cell culture and stimulation

The rat basophilic leukemia RBL-2H3 cell line was cultured as previously described (1). For exosome purification RBL-2H3 cells were incubated with 0.2 μg/mL anti-DNP IgE (SPE-7) (Sigma-Aldrich, St Louis, Missouri, USA) overnight and then stimulated or not with 1 μg/mL DNP-HSA (Sigma-Aldrich) for 6 hours at 37°C in E-MEM supplemented with 10% vesicle-depleted FCS obtained by ultracentrifugation at 100,000*g* for 3 hours at 4°C in a Beckman ultracentrifuge (Beckman Coulter, Brea, CA, USA).

Mouse BMMCs were obtained by *in vitro* differentiation of cells flushed from femurs of female 4-6 weeks old C57BL/6 and cultured in RPMI (Euroclone) supplemented with 10% Fetal Calf Serum (FCS), 25 mM β-mercaptoethanol, 30 ng/mL recombinant mouse Interleukin (IL)-3 (PeproTech, London, United Kingdom), 25 ng/mL recombinant mouse Stem Cell Factor (PeproTech), 2 mM glutamine and antibiotics.

After 4-6 weeks BMMCs were more than 95% pure, as assessed by FACS analysis after staining for FcεRI and CD117.

For exosome purification BMMCs were incubated with 1 μg/mL of IgE for 6 hours at 37°C in RPMI supplemented with 10% FCS and 3 ng/mL IL-3. After washing, cells were stimulated or not overnight with 100 ng/mL DNP-HSA in the presence of 10% exosome-depleted serum and 3 ng/mL IL-3.

Purification of exosomes

Nanovesicles were isolated from MC culture supernatant as previously reported (2), with some modifications. Cell culture media were subjected to two successive centrifugations at 300g for 5 minutes and 1000g for 30 minutes to eliminate cells and debris, followed by a centrifugation at 10,000g for 30 minutes to remove microvesicles. Nanovesicles were then pelleted by ultracentrifugation at 100,000g for 70 minutes, and the pellet was washed.

To purify exosomes from human samples, after serum filtration with 0.2 μm filter, vesicles were isolated by ultracentrifugation (100,000g for 70 minutes at 4°C) and washed twice with PBS. As alternative method, after serum filtration and a first step of ultracentrifugation, pellets were resuspended in PBS and vesicles were immuno-isolated through incubation with CD63-dynabeads (Invitrogen, Carlsbad, California, USA) for 18-22 hours at 4°C under gentle

agitation. Beads were washed twice with 1% BSA in PBS (Sigma-Aldrich, St Louis, Missouri, USA), and immediately used for flow cytometric analysis.

Ultrastructural analysis and immunoelectron microscopy

Trasmission Electron Microscopy (TEM) of MC-isolated nanovesicles was performed as previously described (2). Digital images were taken with Mega View imaging software.

Dynamic light scattering (DLS)

Vesicle size and concentration were measured by DLS using a Zeta-sizer Nano ZS90 spectrometer (Malvern, UK) equipped with a 5 mW HeNe laser (wavelength λ D 632.8 nm) and a non-invasive back-scattering optical setup (NIBS). The detected intensity for each sample was processed by a digital logarithmic correlator, which calculates a normalized intensity autocorrelation function. By fitting the correlation curve to an exponential function, the diffusion coefficient (D) could be estimated. D was converted into an effective hydrodynamic diameter DH through the Stokes–Einstein equation: DH=KBT/(3πηD), where KBT is the system's thermal energy and η represents the solvent viscosity. A sample volume of 40 μL has been used with solvent-resistant micro cuvettes (ZEN0040, Malvern, Herrenberg, Germany). The count rates obtained were then corrected for the attenuator used. Vesicle concentration was calculated by means of a DLS-based non-invasive tool (3). Briefly, the size distribution by intensity $P_I(r)$ of the investigated sample solution is measured by using the regularized Laplace inversion of the detected intensity auto-correlation function. $P_I(r)$ is made independent of the instrumental set-up by normalizing the total scattering intensity to the Rayleigh ratio of the sample R(q), which take into accounts the instrumental photon count rate. Then, the number-weighted radius distribution of vesicles, $P_N(r)$, is obtained as:

$$P_N(R) = \frac{P_I(R)}{kP(qr)[M(r)]^2} \quad (1)$$

Where P(qr) is the form factor of an exosome-like vesicle (approximated as a core-shell spherical system), k is the optical constant and M(r) is the vesicle mass. In the calculation, we used the refractive index (RI) of plasma membrane (n_L = 1.46) for the vesicle shell and the RI of cytoplasm (n_C=1.38) for the vesicle core. The optical constant is given by:

$$k = \frac{4\pi^2 n_0^2}{\lambda_0^2 N_A}\left(\frac{dn}{dc}\right)^2 \quad (2)$$

Where N_A is the Avogadro's number, λ_0 is the incident wavelength, n_0 is the RI and dn/dc is the RI increment of the sample. The vesicle mass was calculated as follows:

$$M(r) = \frac{4}{3}\pi\delta_{LB}[r^3 - (r - \delta r)^3] \quad (3)$$

Where δ_{LB} = 1.12 g/cm^3 is the density of a lipid bilayer of thickness δr, with composition equivalent to that of plasma membrane and r is the vesicle radius. By combining Equations 1-3, the number-weighted radius distribution, $P_N(r)$, is finally calculated and the integral of $P_N(r)$ provides the vesicle concentration (number/mL).

Flow cytometry analysis

Murine vesicles (10 μg) were passively adsorbed on 10 μL of 4 μm Aldehyde/Sulfate Latex Beads (Life Technologies, Carlsbad, California, USA) for 18-22 hours at 4°C in PBS and then the reaction was stopped with 100 mM glycine for 30 minutes. Vesicle/beads complexes were labelled with PE-conjugated anti-CD81 Ab (Abcam, Cambridge, United Kingdom), FITC-conjugated anti-IgE (R35-72, BD Biosciences), AlexaFluor 488 anti-DNP (Life Technologies, Carlsbad, California, USA) or ctrl-isotype Ig diluted in PBS 0.5% exosome-free FCS.

For human analysis vesicles were isolated with CD63-dynabeads and stained with PE-conjugated anti-CD63 Ab (H5C6, BioLegend, San Diego, California, USA) and BV510-conjugated anti-FcεRI-α chain (AER-37/CRA-1, BioLegend).

Vesicle/beads complexes were analyzed using a FACSCanto II (BD Biosciences). Data analysis was performed using the FlowJo program.

Small interfering RNA (siRNA)

Hrs-siRNA was performed by electroporation, as previously described (1). After 24 hours from transfection, cells were processed for Western blotting and vesicle purification.

Vesicles uptake

Nanovesicles were labelled using the red fluorescent dye PKH26 (Sigma-Aldrich), washed with PBS by ultracentrifugation and incubated with unloaded or IgE-loaded RBL-2H3 cells (10 μg of exosomes/10^6 cells) for 1 hour at 37°C. Cells were washed and acquired using a FACSCanto II (BD Biosciences, San Jose, CA).

To inhibit endocytosis, RBL-2H3 cells were pretreated with 100 μM dynasore (Sigma-Aldrich) for 30 minutes and vesicle uptake was performed in the presence of the inhibitor.

For confocal microscopy, IgE-sensitized RBL-2H3 cells were plated in complete medium on glass slides coated with 2% gelatin and incubated with 1 μg PKH26 vesicles for 1 hour at 37°C. Cells were fixed with 4% paraformaldeyde solution and counterstained with DAPI. Slides were mounted and images were acquired and processed as previously described (4).

Western blotting

Cells were lysed, as previously described (1) while vesicles were lysed in a buffer containing 0.5% Triton, 300 mM NaCl, 50 mM Tris pH 7.5, 10 μg/mL aprotinin, 4 μg/mL leupeptin, 1mM PMSF, 5 mM NaF and 1 mM Na_3VO_4. Lysates were resolved by SDS-polyacrylamide gel (PAGE) and electro-blotted onto nitrocellulose membranes as described (1).

The following Abs were used: mouse anti-CD63 mAb (AD1, Abcam, Cambridge, UK), rabbit anti-calreticulin polyclonal Ab (PA-3-900, ThermoScientific/Affinity Bioreagents, Golden, Colorado, USA) and rabbit anti-Hrs (M-79) Ab (Santa Cruz Biotechnology, Dallas, Texas, USA). Anti-rat FcεRI β chain mAb was kindly provided by Dr. J.P. Kinet (Beth Israel Deaconess Medical Center, Boston, USA).

Densitometric analysis was performed using FIJI Image J software.

Functional assays

RBL cell degranulation assay was performed as previously described (5).

Mouse BMMCs were sensitized with 1 μg/mL IgE for 1 hours 37°C in complete media, washed twice with Tyrode's buffer (5) and treated with different doses of DNP-HSA or with vesicles for 30 minutes at 37°C. The enzymatic activity of β-hexosaminidase in supernatants and cell pellets was measured as previously described (5).

For Luminex assay, mBMMCs were sensitized with 1 μg/mL IgE in cytokine-free medium at 37°C for 1 hour and stimulated or not with DNP-HSA or vesicles for 6 hours. Cell-free supernatants were subjected to quantitative analysis of IL-6, IL-13 and TNF-α using a Luminex Mouse Magnetic Assay (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's instructions. Analysis was achieved reading a minimum of 50 beads per cytokine on Bio-Plex® MAGPIX™ Multiplex Reader using xPONENT® software.

In some experiments, after 1 minute of stimulation, cells were treated with DNP-Lys (50 μM).

IgE evaluation

Human sera were collected at the Division of Allergy ("Sapienza" University of Rome, Policlinico Umberto I, Rome) according to protocols approved by the Ethic Committee (RIF.CE 5086) and informed consent was obtained from all participants.
Human sera were first screened for total IgE levels by ImmunoCAP Total IgE system (Phadia, ThermoFisher scientific, Carlsbad, California, USA).
Vesicles were isolated from human sera and the associated IgE was measured by Abcam's IgE ELISA Kit (ab195216) following the manufacturer's protocol.

Statistical analysis

Statistical significance between two groups was determined by performing two-tailed, paired Student's t-test. Differences between multiple groups were analyzed with two-way analysis of variance (ANOVA) with Tukey post-test correction. Prism 7 software (GraphPad Software, San Diego, Calif) was used. Graphs show mean values, and all error bars represent the SD.

References

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FIGURES AND FIGURE LEGENDS

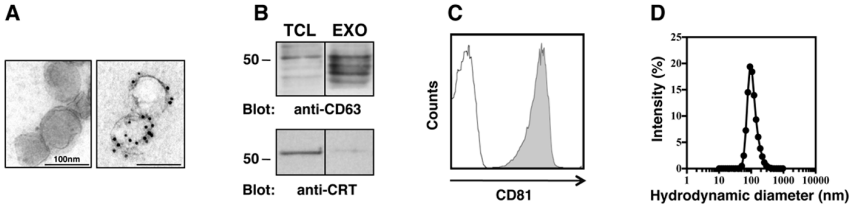
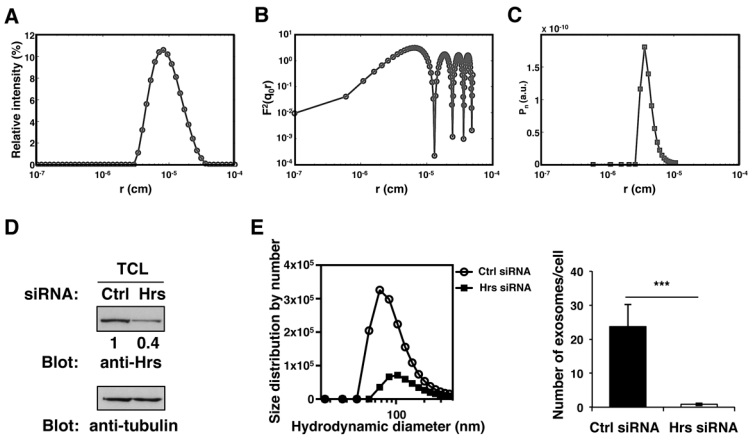


Figure S1. Phenotypical characterization of nanovesicles released by RBL-2H3 cells. Exosomes were purified from RBL-2H3 cell culture supernatants. Transmission electron microscopy (**A**, left panel) and immunogold labelling using anti-CD63 Ab (**A**, right panel). Results are representative of three independent experiments. **B**, Western blot analysis on total cell lysates (TCL) or exosome-like fractions (EXO) using Abs against CD63 and calreticulin (CRT), a marker of endoplasmic reticulum. Representative blots of three independent experiments are shown. **C**, Vesicles were passively adsorbed on 4 μm latex beads, labelled with PE-conjugated anti-CD81 (filled histogram) or isotype control (empty histogram) Abs and analyzed by flow cytometry. **D**, Representative exosomal size analysis by dynamic light scattering (DLS).



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210 **Figure S2. Biogenesis of MC-derived nanovesicles requires Hrs.**

211 **A-C**, Calculation of exosome concentration by Dynamic Light Scattering (see Material and
212 Methods). **A**, Representative intensity-weighted size distribution function of vesicles. **B**,
213 Form factors used for the calculation of vesicle concentration. Exosome-like vesicles are
214 approximated to core-shell spherical lipid bilayer vesicles. The refractive index of the shell is
215 set equal to that of the plasma membrane (nL = 1.46) and the refractive index of cytoplasm
216 (nC= 1.38) is used for the vesicle core. **C**, Number-weighted size distribution, Pn calculated
217 as described in Material and Methods. **D** and **E**, RBL-2H3 cells were transfected with Hrs-
218 siRNA or with Ctrl-siRNA. **D**, After 48 hours total cell lysates were analyzed by Western
219 blotting with the indicated Abs. The relative amount of Hrs, normalized to tubulin, was
220 referred to the control sample. **E**, The size distribution of exosome-like vesicles derived from
221 Ctrl- or Hrs-siRNA transfected cells was measured by DLS. The number of exosomes per
222 cell is shown as mean ± SD of three independent measures. ***$p<0.001$, Student's t-test.

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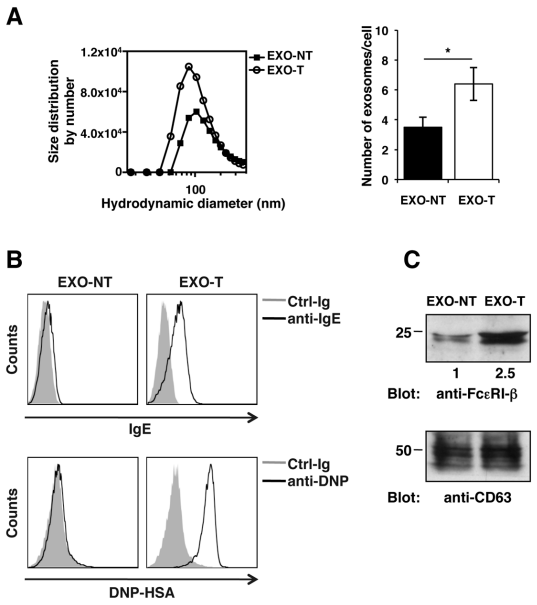

Figure S3. Antigen stimulation of RBL-2H3 cells increases vesicle release and promotes the delivery of IgE and antigen into exosome-like vesicles.

Nanovesicles were isolated from supernantants of RBL-2H3 cells loaded with anti-DNP IgE mAb (SPE-7) and stimulated or not with the multivalent antigen, DNP-HSA. **A**, Vesicle size distribution was evaluated by DLS (left panel) and the number of exosomes per cell is shown as mean ± SD of three independent measures (right panel). *$p<0.05$, Student's t-test. **B**, Equal amount (10μg) of exosome-like vesicles released by unstimulated (EXO-NT) or antigen-stimulated MCs (EXO-T) was passively adsorbed on 4μm latex beads. IgE and antigen expression was evaluated by FACS analysis using FITC-conjugated anti-IgE or Alexa 488-conjugated anti-DNP (empty histograms). Filled histograms represent staining with isotype control Abs (Ctrl Ig). Results are representative of three independent experiments. **C**, EXO-NT and EXO-T released by RBL-2H3 cells were lysed, and equal amount of proteins (20 μg) were separated by electrophoresis. FcεRI-β chain and CD63 were analyzed by Western blotting. The relative amount of FcεRI-β chain was normalized to CD63 and the fold difference of FcεRI-β chain in the stimulated compared to the unstimulated sample is indicated below the lanes in the upper panel.

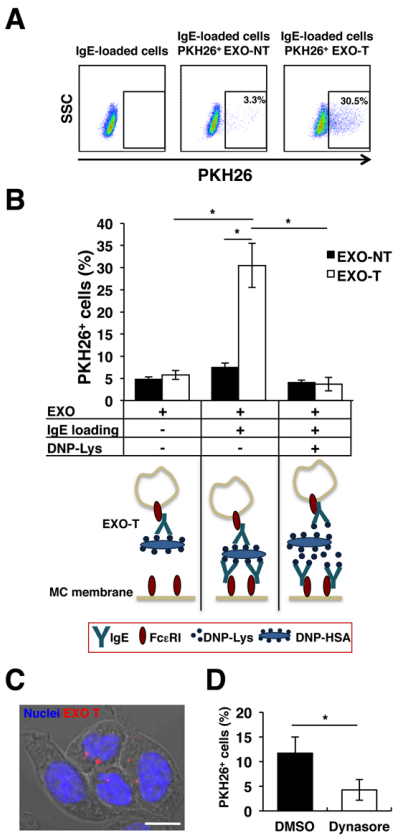

Figure S4. Vesicles endowed with antigen are up-taken by MCs.

A and B, Exosome-like vesicles purified from unstimulated (EXO-NT) or stimulated (EXO-T) RBL-2H3 cells were labelled with PKH26 red fluorescent dye and incubated with IgE-unloaded or loaded cells for 1 hour. Where indicated, 50 μM of monovalent antigen (DNP-Lys) was added to the culture 1 minute after the vesicles. **A**, The percentage of red positive cells was analyzed by flow cytometry on SSC-A/PKH26 dot plot by using cells w/o vesicles as negative control. **B**, Means ± SD of three independent experiments together with a schematic representation of different experimental conditions. In particular, the high-affinity monovalent Ag DNP-Lysine (DNP-Lys) works as a scaffold displacing the IgE-bound multivalent Ag DNP-HSA and blocking additional binding between IgE and DNP-HSA. *p<0.05, one-way ANOVA with Tukey's multiple comparison test. **C**, IgE-loaded RBL-2H3 cells were incubated with PKH26-labelled EXO-T for 1 hour and then extensively washed, fixed, counterstained with DAPI (blue) and visualized by confocal microscopy. Images were acquired using 60X/1.35NA oil immersion objective and shown as single optical slice. Scale bar = 10 μm. **D**, RBL-2H3 cells pretreated with 50 μM dynasore or vehicle alone (DMSO) were incubated with PKH26-labelled EXO-T for 1 hour. The percentage of PKH26-positive cells was analyzed by flow cytometry, as in panel **A**. Data are represented as mean ± SD of three independent experiments. * p<0.05, Student's t-test.

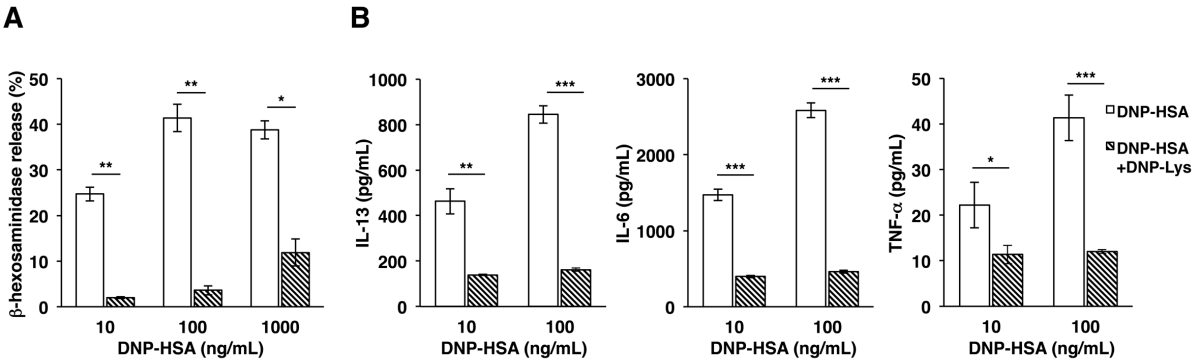

Figure S5. BMMC degranulation and cytokine production induced by antigen stimulation.

A, IgE-loaded mBMMCs were incubated with different doses of DNP-HSA. β-hexosaminidase release was measured in culture supernatants. Where indicated, 50 μM DNP-Lys was added upon 1 minute of stimulation. **B**, Mouse BMMCs were sensitized by the addition of IgE and then stimulated for 6 hours with different doses of DNP-HSA. Where indicated, 50 μM DNP-Lys was added upon 1 minute of stimulation. Supernatants were collected, and cytokines were measured by multiplex cytokine assay. Dashed lines indicate the cytokine amounts released by unstimulated cells.

All data are presented as the mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, one-way ANOVA with Tukey's multiple comparison test.

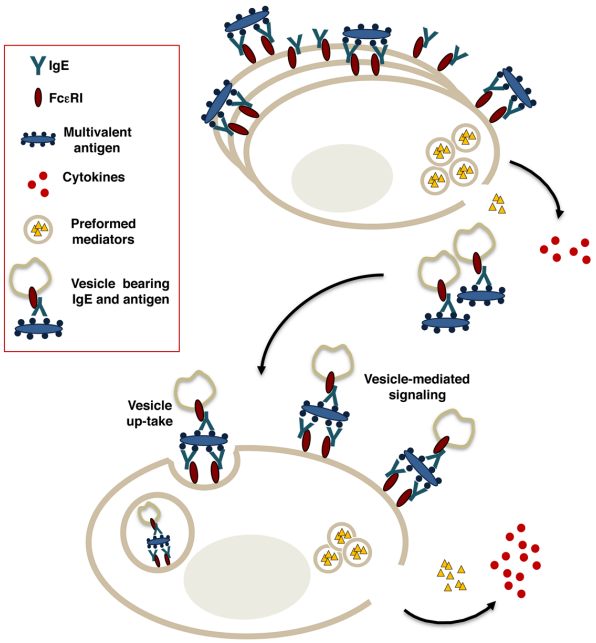

Figure S6. Schematic representation of MC activation induced by FcεRI/IgE/Ag carrying exosomes.

Upon MC stimulation by multivalent antigen, internalized FcεRI/IgE/Ag complexes can be released in exosome-like vesicles. Those nanoparticles are easily up-taken by sensitized MCs and induce their activation, thus representing a potential mechanism of allergic reaction amplification.

Table S1. IgE and FcεRI α chain in EVs from human donors

ATOPIC DONORS	Gender/Age	Total IgE level (kU/L)	EV-associated IgE (pg/mL)	FcεRI α chain (MFI)
1	M/42	1819	190	nd
2	M/44	4719	2000	nd
4	M/33	4706	1800	nd
5	F/47	1033	300	nd
6	F/34	2213	600	321
7	F/69	1217	90	nd
8	M/18	1276	nd	162
9	M/53	>5000	nd	320
10	M/31	>5000	nd	267
11	F/45	>5000	nd	229
12	F/52	>5000	nd	241
13	M/25	980	911	nd
14	M/13	1914	932	nd
15	F/51	1640	160	nd
16	M/61	1342	114	nd
17	M/54	1235	85	nd
18	F/52	1083	1012	nd
NON-ATOPIC DONORS	Gender/Age	Total IgE level (kU/L)	EV-associated IgE (pg/mL)	FcεRI α chain (MFI)
1	M/43	2.57	0	nd
2	F/53	2	0	nd
3	F/61	2.3	0	nd
4	F/19	3.1	0	nd
5	F/22	20.8	nd	135
6	F/45	0	nd	118
7	F/26	30.2	nd	114
8	F/21	0	0	123
9	M/37	7.3	0	120

***MFI*, mean fluorescence intensity; *nd*, not determined.**