A highly sensitive bioluminescent method for measuring allergen-specific IgE in microliter samples

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Supplementary methods

Design and synthesis of plasmid encoding the anti-IgE nanobody-luciferase tandem

Rvr:5'GGTATATCTCCTTCTTAAAGTTAAAC3', Eurofins) using a Q5 DNA polymerase, dNTP mix (New England BioLabs). PCR product was purified by electrophoresis on agarose gel (1%, Macherey Nagel). Purified pET23 vector and the synthetic gene were assembled (pET23-*kaz*) using NEBuilder HiFi assembly master mix (New England BioLabs).

The Fcc3 IgE–binding moiety (Kd= 1.4 nM) is from a humanized heavy chain antibody of the camelid species llama (*lama glama*) selected against IgE (single-domain antibody, sdAb026)². The gene *sdab026* has been synthetized by Eurofins with a flanking region corresponding to the pET23-*kaz* sequence. Synthetic gene *sdAb026* has been amplified with the corresponding forward and reverse oligonucleotides (Fwd: 5'ATGGTCTTCACACTCGAAGATTTC3'; Rvr: 5'CATGGTATATCTCCTTCTTAAAGTTAAA3'; Eurofins) using a Q5 DNA polymerase and dNTP mix. PCR products were purified by electrophoresis on agarose gel. Purified pET23-*kaz* vector and the synthetic gene *sdAb026* were assembled using NEBuilder HiFi assembly master mix. The assembled products (5 μ L) were used to transform NEB 5-alpha competent *E. coli* and grown overnight on LB/Agar/ampicillin in a Petri dish. Isolated colonies were grown in liquid medium, plasmids were isolated and nucleotide sequence was performed to confirm the presence of the *sdabs026-kaz* insert. The full sequence of anti-IgE nanobody luciferase tandem

sdAb026-nanoKAZ ([nanobody anti-IgE]-[luciferase]-[His₆]) is shown in **Supplementary Fig** 1.

Expression, purification and validation of anti-IgE nanobody-Luciferase tandem

pET23-*sdab026-kaz* was used to transform *E.coli* BL21 (DE3) to achieve high expression in *E.coli*. Cells were grown at 18°C and IPTG was added to induce sdAb026-nanoKAZ production. After harvesting the cells by centrifugation (1.5 L), the pellet was resuspended in 50 mM Tris-HCl pH 8.0, 50 mM NaCl with protease inhibitor (Sigma) and lysozyme (0.1 mg/mL). Cells were disrupted by freezing-thawing cycle lysis method. DNase (Sigma-Aldrich) was then added to remove DNA from the sample.

The crude extract was centrifuged 30 min at 15000 g. The supernatant was collected and NaCl (500 mM), Imidazole (20 mM) and Triton (0.1 %) were added. The cleared lysate was loaded on a Hi-Trap 5 mL-column (GE-Healthcare) at 4 mL/min using an AKTA pure chromatography system (GE-Healthcare). The column was washed with 20 volumes of column with a running buffer (50 mM Tris-HCl pH 8.0, NaCl 50 mM, 20 mM imidazole) at 5 mL/min. The sdAb026-nanoKAZ was eluted with a gradient of imidazole from 20 mM to 200 mM in 50 mM Tris-HCl pH 8.0, 50 mM NaCl at 5 mL/min and fractions of 1 mL were collected in a 96-deepwell plate (GE-Heathcare). Catalytic activity of fractions was profiled using a luminometer Hydex by diluting 10⁷ fold the fractions in PBS with 27 μ M of furimazin (imidazo[1,2-*a*]pyrazin-3(7*H*)-ones). The fractions of high activity were pooled, and loaded on a 1 mL HiTrap Q column (GE-Heathcare) equilibrated in 50 mM Tris-HCl pH 8.0, NaCl 50 mM. The protein was eluted in 50 mM MES pH 6.5, 50 mM NaCl at 1 mL/min at 18°C using the AKTA pure chromatography system. The fractions of 500 μ L were collected in 96-deepwell plates and their activities were assayed as described above. The fractions of high activity were pooled. The quality of the purified protein was assessed by loading an aliquot (10 μ L) on a stain-free SDS gel (4–15%)

Mini-PROTEAN® TGX Stain-FreeTM Protein Gels, Bio-Rad. The gel was activated by UV trans-illumination for 5 min (Bio--Gel Doc XR Imaging System). Tryptophan residues undergo an UV-induced reaction with trihalo compounds and produce an imaged fluorescence signal (**Supplementary Fig 1, C**). An UV-spectrum (240-300 nm) was acquired for evaluating the concentration of sdAb026-nanoKAZ (MW 34,726 Da) from the solution absorption at 280 nm ($\varepsilon = 50,780 \text{ M}^{-1}\text{cm}^{-1}$ estimated by the method of Gill and von Hippel (*Analytical Biochemistry*, **182**: 319-326, 1989)). The specific activity is about $4 \cdot 10^{17} \pm 0.5 \cdot 10^{17} \text{ RLU} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$ of sdAb026-nanoKAZ.

IgE LuLISA

White 96-well plates with flat bottoms (Fluoronunc C96 Maxisorp Nunc) were coated with either 10 µg/mL of peanut extract (PE, F171, Greer laboratories), 5 µg/mL of ovalbumin (OVA, Sigma) or 1 µg/mL of Der p 2 (NA-DP2, Indoor Technology) in NaHCO₃ 50 mM buffer, 50 µL/well for 2 hours at room temperature. Wells were emptied and the coating was saturated with bovine serum albumin (Sigma) at 100 µg/mL in PBS for 1 hour at room temperature. Wells were washed four times with 100 µL of PBS/Tween 20 0.1%. Recombinant anti-OVA human chimeric IgE (clone X4A4D12/G9/H8, a kind gift from Arkab), recombinant human anti-Der p 2 IgE (2B12 CH-1, Indoor Technology), recombinant anti-Der p 2 human IgG1 (2B12 CH-2, 2B12, Indoor Technology), recombinant IgG4 (2B12 CH-3, Indoor Technology) or plasma from peanut allergic subjects were diluted in PBS or in a pool of plasma from healthy donors, as indicated. Sample dilutions were incubated 1 hour at room temperature in their respective allergen-coated wells, 50 µL/well. Wells were washed four times with 100 µL PBS/Tween 20 0.1%. Purified sdAb026-nanoKAZ 1 ng/mL (400·10⁶ RLU·s⁻¹·mL⁻¹) in PBS was loaded (50 µL/well) and incubated 30 min at room temperature. Wells were washed four times with 100 μ L of PBS/Tween 20 0.1%. Plates can be stored at this step in PBS until measurements. Just before reading, each of the wells were emptied and loaded with 50 μ L of furimazin (imidazo[1,2-a]pyrazin-3(7H)-ones) at 27 μ M (about 10 times the *K*_M of nanoKAZ). The plate was orbitally shaken for 5 seconds and the collected photons were counted during 1 sec per well and measured 3 times in a plate luminometer (LB960 Centro, Berthold).

IgE ImmunoCAP

ImmunoCAP analysis was performed by Phadia, Thermo Fisher (Uppsala, Sweden and Villebon, France), 120 μ L of plasma per analyzed sample.

IgE ELISA

Clear, flat bottomed, 96-well plates (Maxisorp, Nunc) were coated with either 10 μ g/mL of peanut extract (PE, F171, Greer laboratories), 5 μ g/mL of ovalbumin (Sigma) or 1 μ g/mL of Der p 2 (NA-DP2, Indoor Technology) in NaHCO₃ 50 mM pH 9.5 (Sigma) and incubated for 2 h at room temperature. The plates were then emptied and loaded with BSA 100 μ g/mL (Sigma) in NaHCO₃ 50 mM pH 9.5 (Sigma) and incubated for 1 h for well surface saturation at room temperature. Wells were washed four times with 100 μ L of PBS/Tween 20 0.1%. Recombinant anti-OVA human chimeric IgE (100 ng/mL, clone X4A4D12/G9/H8, a kind gift from Arkab), recombinant human anti-Der p 2 IgE (20 ng/mL, CH1, Indoor Technology), or plasma from peanut allergic subjects (1 μ L) were diluted in PBS (50 μ L/well) and incubated for 1 hour at room temperature in their respective allergen-coated wells. Wells were washed four times with 100 μ L of PBS/Tween 20 0.1%. Then, 50 μ L of goat IgG anti-human IgE conjugated with alkaline phosphatase (dilution 1:700 in PBS, Sigma) were loaded in each well and incubated for 1 hour at room temperature. Wells were washed four times with 100 μ L of place the provide the pro

PBS/Tween 20 0.1%. All wells were emptied and 50 μ L of phosphatase substrate pNPP 1 mg/mL (4-Nitrophenyl phosphate disodium salt, Sigma) were added per well and incubated for 3 h at room temperature or overnight at 4°C. Absorbances were measured at 405 nm with a plate spectrophotometer after 3 or 24 h of incubation (Sapphire, Tecan).

Statistical analysis

Differences in IgE levels between plasma samples from healthy donors and peanut allergic subjects were compared using an unpaired Mann-Whitney U test. P values < 0.05 are considered statistically significant. Correlation analyses between IgE LuLISA and IgE ImmunoCAP were performed using two-tailed non-parametric Spearman's tests.

Supplementary references

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Α ATG GOG GAG GTC CAG TTG CTT GAG AGT GGT GGG GGT CTT GTT CAG OCA GGG GGC TCC CTT CET CTT AGT TET COC CCA TOC CET TTC ACA TTC CEC AAT TAC GAC ATG COG TEG ETC CGA CAA GCT CCC GGC AAA CGC CCT GAG TGG GTA TCG TCT ATT GAT ACG GGG GGA GAT ATA ACA CAC TAC QCA GAC AGT GTG AAG QQG AGG TTC ACA ATC TCT QQG GAC AAC QCA AAG AAC ACC TTA TAC TTG CAG ATG AAT ACC CTG AGA COG GAG GAC ACT CCT GTG TAT TGG TCC CCT ACC GAT GAA GAA TAT GOC CTC GOC CCA AAC GAA TTT GAT TAT TAC GGA CAG GGA ACT CTG GTG ACT GTG TCA TCA OCT OCC CTG GAG ATG GTC TTC ACA CTC GAA GAT TTC GTT OOG GAC TEG CEA CAG ACA ECC EEC TAC AAC CTE GAC CAA ETC CTT GAA CAG EEA ETE TCC AET TTG TTT CAG AAT CTC GOG GTG TCC GTA ACT CCG ATC CAA AGG ATT GTC CTG AGC GGT GAA AAT GOG CTG AAG ATC GAC ATC CAT GTC ATC ATC CCG TAT GAA GGT CTG AGC GOC GAC CAA ATG GOC CAG ATC GAA AAA ATT TTT AAG GTG GTG TAC CCT GTG GAT GAT CAT CAC TTT AAG GTG ATC CTG CAC TAT GOC ACA CTG GTA ATC GAC GOG GTT ACG CCG AAC ATG ATC GAC TAT TTC GGA COG CCG TAT GAA GOC ATC GCC GTG TTC GAC GOC AAA AAG ATC ACT GTA ACA GOG ACC CTG TGG AAC GOC AAC AAA ATT ATC GAC GAG COC CTG ATC AAC COC GAC GOC TCC CTG CTG TTC CGA GTA ACC ATC AAC GGA GTG ACC GGC TGG CGG CTG TGC GAA CGC ATT CTG GCG CTC GAG CAC CAC CAC CAC CAC TGA

10	20	30	40	50	60
MAEVQLLESG	GGLVQPGGSL	RLSCAASGFT	FGNYDMAWWR	QAPGKRPEW	SSI DTGGDI T
70	80	90	100	110	120
HYADSVKGRF	TI SRDNAKNT	LYLQMNSLRP	EDTAVYWCAT	DEEYALGPNE	FDYYGQGTLV
130	140	150	160	170	180
TVSSAAALEM	VFTLEDFVGD	WRQTAGYNLD	QVLEQGGVSS	LFQNLGVSVT	PI QRI VLSGE
190	200	210	220	230	240
NGLKIDIHVI	I PYEGLSGDQ	MGQI EKI FKV	VYPVDDHHFK	VI LHYGTLVI	DGVTPNM DY
250	260	270	280	290	300
FGRPYEGI AV	FDGKKI TVTG	TLWWGNKI I D	ERLI NPDGSL	LFRVTI NGVT	GWRLCERI LA

20 14

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MW 2 7 8 9 10 3 4 5 6 94 🕨 67 43 30

Figure S1. Sequence and characterization of the anti-IgE nanobody-luciferase tandem (sdAb026-KAZ). (A and B) DNA sequence of sdAb026-kaz (A), and corresponding amino acid sequence of sdAb026-KAZ (B). SdAb026 is a humanized heavy chain antibody of llama (singledomain antibody SdAb, nanobody or V_HH) directed against human IgE. KAZ is an optimized sequence of the catalytic domain of the luciferase from Oplophorus gracilostris. In red: sdAb026; In black: KAZ; In blue: His₆-Tag. (C) sdAb026-KAZ was produced in E. coli and purified using HiTrap chromatography column eluted in phosphate 50mM, imidazole 100mM, NaCl 150mM (lane 2), and DEAE eluted in MES 50mM NaCl 150mM pH 6.5 (successive 1mL-fractions from lanes 3 to 10). The purity and size of sdAb026-KAZ was assessed on SDS gel. The estimated molecular weight (MW) of sdAb026-KAZ calculated from sequence is 34.1kD. Protocols are detailed in supplementary ref. 3.



Figure S2. Correlation between LuLISA bioluminescent signal and sigE concentration in international units. Recombinant human anti-ovalbumin (OVA) IgE were diluted in PBS at the indicated concentrations and incubated with plate-bound OVA. Bioluminescent detection of antibody levels was performed by LuLISA using the anti-IgE sdAb026-nanoKAZ. RLU: relative light unit.



Figure S3. Comparison between IgE LuLISA and IgE ELISA. Recombinant anti-ovalbumin (OVA) IgE was diluted in PBS at the indicated concentrations. Levels of OVA sIgE were assessed in aliquots from the same dilution sample using LuLISA or ELISA. RLU: relative light unit.



Figure S4. Influence of the concentration of the anti-IgE nanobody-luciferase tandem on bioluminescent detection of IgE by LuLISA. Plasma from a peanut allergic subject ("PN allergic plasma"; peanut-specific IgE ImmunoCAP value: 2186 kU/L) was diluted 2000 times in PBS and incubated in triplicate with plate-bound peanut extract. Plasma from a healthy donor ("HD plasma") diluted 2000 times in PBS or PBS alone were used as controls. Bioluminescent detection of peanut-specific IgE levels was performed by LuLISA at the indicated concentrations of anti-IgE nanobody-luciferase tandem (sdAb026-nanoKAZ). Each point represents average values from triplicates. Area in grey shows values obtained at the sdAb026-nanoKAZ concentrations used for all other experiments in this study. RLU: relative light unit.



Figure S5. Detection of peanut sigE by LuLISA using dilution series of plasma from peanut allergic subjects. Plasma specimens from six peanut allergic subjects were diluted in PBS at the indicated concentrations and incubated with plate-bound peanut extract. Bioluminescent detection of peanut sIgE levels was performed by LuLISA. Red arrows indicate plasma dilution used in Figure 2A. Grey areas show the linear range of detection of the peanut sIgE LuLISA. RLU: relative light unit.

patient's ID#	Peanut DBPCFC cumulated tolerated dose (mg)	Skin prick test peanut wheal diameter (mm)	ImmunoCAP Total IgE (IUA/L)	ImmunoCAP peanut specific IgE (IUA/L)	ImmunoCAP Ara h 1 sIgE (IUA/L)	ImmunoCAP Ara h 2 sIgE (IUA/L)
P003	75	7.5	985	4.27	<0.1	2.72
P004	175	15.5	470	3.82	<0.1	3.63
P006	375	19	243	7.67	<0.1	5.25
P013	275	8.5	274	30.4	<0.1	0.37
P025	75	20	410	4.18	<0.1	3.03
P030	275	8.5	35	6.49	<0.1	3.54
P033	375	4	550	1.65	<0.1	<0.1
P055	25	11	333	3.58	<0.1	1.54
P059	5	16.5	449	1.46	<0.1	1.29
P060	75	8	240	0.41	<0.1	0.2
P067	5	10.5	73	0.79	0.5	<0.1
P070	5	19.5	443	13.6	<0.1	10.3
P085	25	12	316	2.89	<0.1	1.71
P097	5	14.5	281	5.43	<0.1	1.72
P102	5	5	87	0.62	<0.1	<0.1
P103	5	17.5	102	0.95	<0.1	0.79
P109	0	6	242	25	<0.1	0.24
P111	0	23	1529	1.1	<0.1	1.25

Table S1. Clinical data from all peanut allergic subjects with Ara h 1 and/or Ara h 2 slgE below detection limit by ImmunoCAP (0.1 kU_A/L). DBPCFC: double-blind, placebo-controlled food challenge.