

LETTER

Nanobody-based human antibody formats act as IgE surrogate in hymenoptera venom allergy

To the Editor,

For the canonical antibody isotypes, antigen recognition is driven by the variable regions of both heavy and light chains. In contrast, single-domain antibodies or nanobodies (nb) are the antigen-binding moiety of heavy chain only antibodies occurring in camelid species and cartilaginous fish.^{1,2} Their small-size, high-yield production, and stability render nanobodies versatile building blocks for the development of binders and unorthodox activatory or inhibitory multi-domain derivatives.³ In the allergological context, single nanobodies were used as anti-IgE molecules and as binding moiety for allergens.^{4,5}

Our aim was to generate allergen-specific nanobodies and establish a nanobody-based IgE format in the context of hymenoptera venom allergy (Figure 1A,B). In venom-allergic patients, the IgE response is typically directed to a set of major and minor allergens.⁶ The best-characterized and most abundant honeybee venom (HBV) allergens are phospholipase A2 (Api m 1) and hyaluronidase (Api m 2) (Figure S1A). Therefore, native Api m 1 and recombinant Api m 2 were used for llama immunization (Figure S1B,C). Immune libraries were generated from peripheral blood mononuclear cells. Enrichment by phage display and immunoreactivity of individual phage clones was shown by ELISA (Figure S2). After expression and purification from bacterial supernatant, the nanobodies showed the expected molecular masses in SDS-PAGE and immunoreactivity to their target in ELISA (Figure S3A–C).

Exemplary nanobodies against Api m 1 (AM1-1 and AM1-3) and against Api m 2 (AM2-A1 and AM2-C2) were then converted into homodimeric IgE formats by fusion to IgE CH2-4 domains. After purification from supernatant of stably transfected HEK293 cells, SDS-PAGE and immunoblotting analyses corroborated proper dimerization of the nb-IgE (Figure 1C, Figure S4). Expression yields

of 10–20 mg/L pointed at a favorable performance of the nb-IgE in mammalian hosts as compared to the limited yields often observed for entire IgE antibodies.⁷ The nb-IgEs remained reactive to both their particular target allergen and the FcεRIα in ELISA (Figure 1D).

The antibodies exhibited sIgE reactivity in Euroline assays to HBV and individual allergens without cross-reactivity to other HBV or yellow jacket venom allergens (Figure 2A,B). Furthermore, individual nb-IgE clones were applied on the ImmunoCAP test system. Concentration-dependent sIgE reactivity was detected for Api m 1, Api m 2, and HBV (Figure 2C) corresponding well to the total IgE levels. The sIgE reactivity against Api m 1 was found highly comparable to that of HBV. In contrast, a clearly reduced IgE reactivity to HBV compared with the component Api m 2 pointed toward a lower sensitivity of HBV for patients with sensitization to Api m 2 possibly due to a limited accessibility of immunoreactive Api m 2 in the test. Furthermore, an oligoclonal artificial human serum was established by combining the 4 nb-IgE against Api m 1 and Api m 2 in equimolar concentration. Assessment of sIgE reactivity verified the added sIgE levels for HBV, Api m 1, and Api m 2 (Figure 2D). Notably, the reduced sIgE level for HBV reflected the reduced reactivity of the individual Api m 2-specific nb-IgE to HBV. Hence, artificial sera comprising molecularly defined IgE surrogates of adjustable concentration might replace human sera in applications such as assessment of diagnostic test systems, round-robin tests, and diagnostic and therapeutic extracts, but also mechanistical analyses in basic research.

In summary, we have established nanobodies as a toolbox for the generation of important downstream formats like the novel nb-IgE. This platform also paves the way for the generation of other formats including blocking IgG formats, and hence, has the potential to provide advanced tools for diagnostics, functional analyses, and interventional studies.

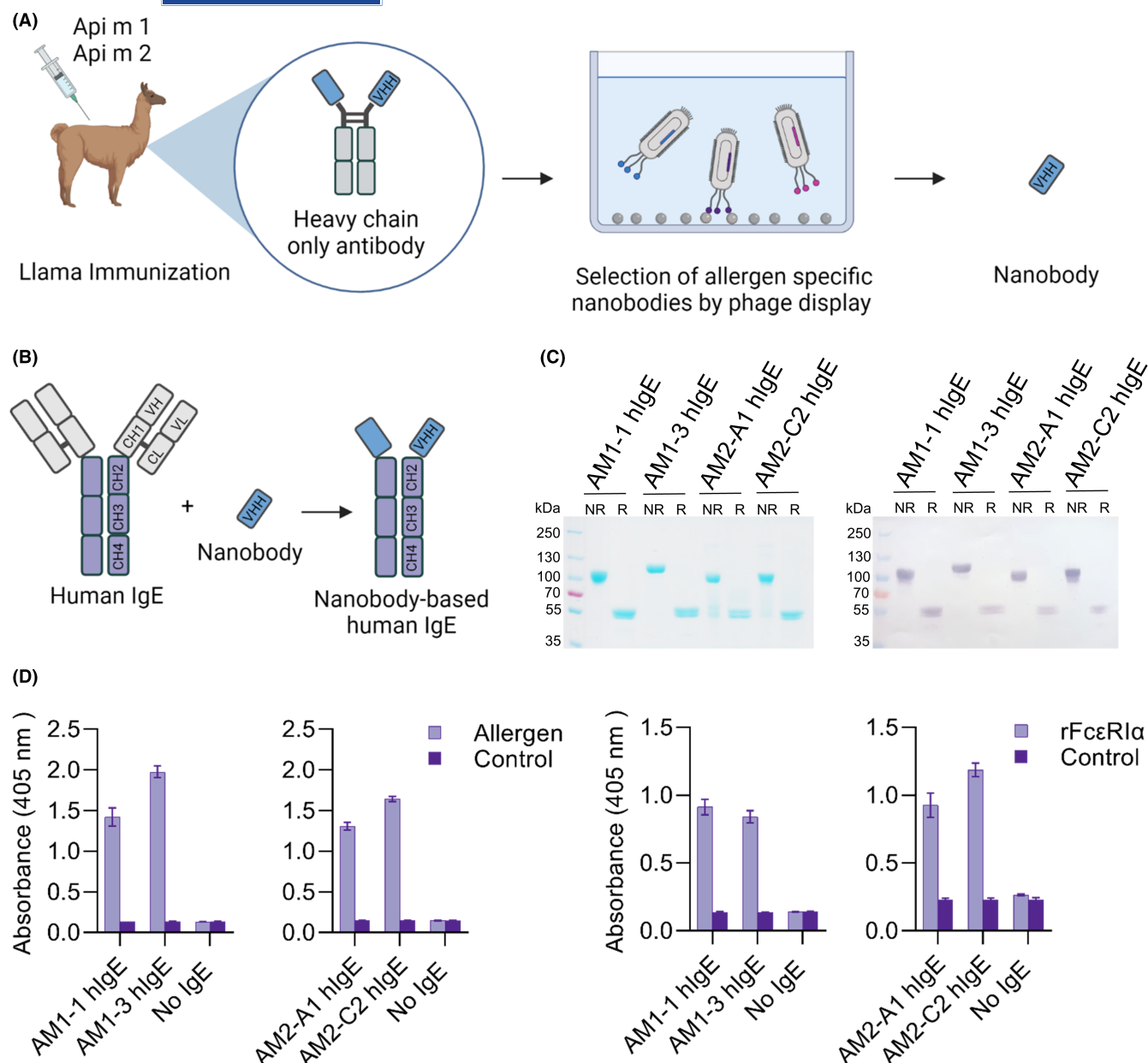
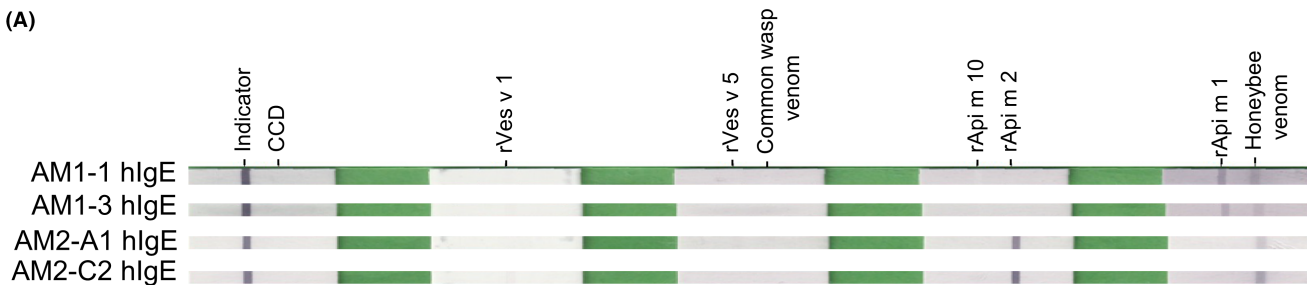


FIGURE 1 Generation of nanobody-based human IgE against nApi m 1 and rApi m 2 (A, B) Schematic representation of the nanobody generation. Upon immunization of llamas with nApi m 1 and rApi m 2, immune repertoire libraries were generated and selected by phage display. Nanobody-based human IgEs (nb-IgEs) were then generated by fusion of obtained allergen-specific nanobodies to the Fc domains of human IgE. (C) SDS-PAGE and immunoblotting analysis of purified nb-IgEs under non-reducing (NR) and reducing (R) conditions. The recombinant nb-IgEs were detected using an anti-IgE antibody conjugated to alkaline phosphatase. (D) The immunoreactivity of purified nb-IgEs was further assessed in ELISA. Binding to immobilized allergen or FcεRIα was detected using an anti-IgE antibody conjugated to alkaline phosphatase. Data are mean ± s.d. of triplicates

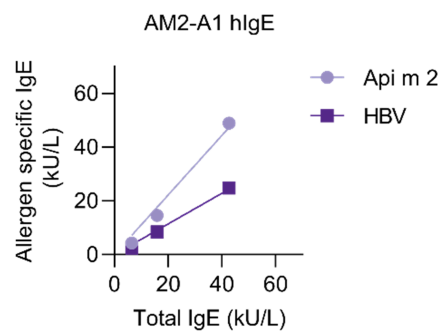
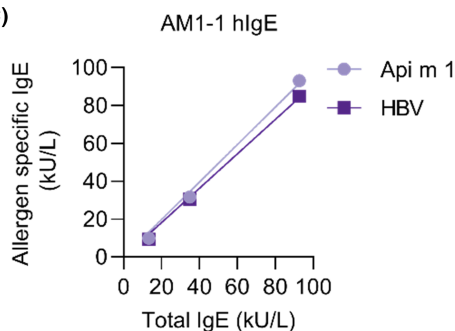
(A)



(B)

	CCD	rVes v 1	rVes v 5	Common wasp venom	rApi m 10	rApi m 2	rApi m 1	Honeybee venom
AM-1-1 hlgE	< 0,35	< 0,35	< 0,35	< 0,35	< 0,35	< 0,35	15,41	8,46
AM1-3 hlgE	< 0,35	< 0,35	< 0,35	< 0,35	< 0,35	< 0,35	10,45	2,72
AM2-A1 hlgE	< 0,35	< 0,35	0,35	< 0,35	< 0,35	69,09	< 0,35	10,45
AM2-C2 hlgE	< 0,35	0,52	<0,35	< 0,35	< 0,35	88,11	< 0,35	33,25

(C)



(D) Oligoclonal serum substitute

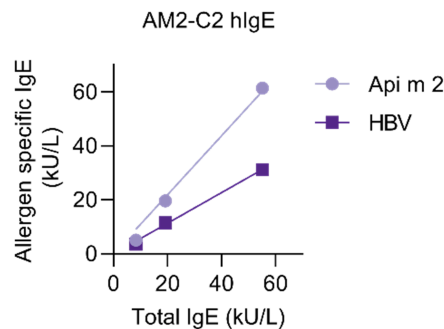
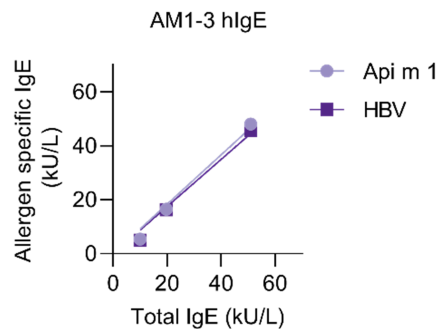
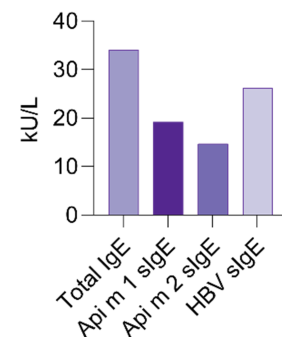


FIGURE 2 IgE measurements on diagnostic platforms. The immunoreactivity of purified nb-IgE was further assessed in commercial test platforms. (A and B) Euroline assay. (C) ImmunoCAP measurements of tIgE and sIgE were performed for dilution series of the four different nb-IgEs. (D) ImmunoCAP measurements of an oligoclonal serum substitute comprising the four different nb-IgEs. Values are single measurements

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KEYWORDS

allergens, anaphylaxis, IgE, insect venom, nanobodies

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CONFLICT OF INTEREST

A patent application covering part of the manuscript has been filed by JBA, CS, MM, and ES. The other authors have no financial conflict of interest and nothing to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

1 **Supplementary data Aagaard et al.**

2

3 **Material**

4 Native Api m 1 and honeybee venom were purchased from Latoxan.

5

6 **Recombinant expression of Api m 2**

7 The recombinant production of insect venom allergens was performed in Sf9 insect cells as
8 described earlier [1]. In brief, cryostocks of Sf9 cells infected with recombinant baculovirus were
9 used to infect Sf9 cells ($1.5\text{--}2 \times 10^6$ cells/mL) in a 2000 mL suspension flask (400 mL suspension
10 culture) according to recommendations of the manufacturer. For protein production, the cells were
11 incubated at 27°C with agitation at 110 rpm for 120 h. The supernatant of baculovirus-infected cells
12 was collected, adjusted to pH 8 and centrifuged at 4000 g for 30 min. The centrifugate was applied
13 to a nickel-chelating affinity matrix (Ni-NTA-excel agarose, GE Healthcare). After washing with
14 NTA-binding buffer (50 mM sodium phosphate pH 8, 500 mMNaCl, 15 mM imidazole), the
15 recombinant protein was eluted with NTA-binding buffer containing 300 mM imidazole. Quality
16 and purity of the obtained protein was assessed by SDS-PAGE and immunoblotting.

17

18 **Library generation and selection**

19 Llamas were immunised with in total 400 µg of the proteins according to manufacturer's
20 protocols (Capralogics, 4 intradermal injections over 73 days in Freund's adjuvant). Peripheral
21 blood mononuclear cell mRNA was prepared from Trizol-solubilized samples after extraction with
22 chloroform and precipitation with ice-cold isopropanol. The cDNA was generated using constant
23 region specific oligonucleotides. PCR was carried out using primers annealing to the 5'- and 3'-end
24 of the nanobody. Nanobody encoding genes were then amplified carrying the restriction sites for
25 SfiI and XhoI, introduced into the phagemid vector via SfiI and XhoI and electroporated into *E. coli*

TG1. The selection of repertoires was essentially performed according to established protocols [2]. Briefly, microwells coated with the antigen and blocked with 30 g/L PBS/2% milk powder (MPBS) were incubated with 100µl of 30 g/L MPBS containing 10^{12} to 10^{13} phages. After incubation, washing, and acidic elution of bound phages, reinfection of *E. coli* ER2738 and rescue by M13KO7 helper phage, phages were subjected to the next round of selection. Immunoreactivity of phages was assessed in ELISA with anti-M13-horseradish peroxidase conjugate (GE Healthcare) and 2,2-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) di-ammonium salt for detection.

Recombinant expression of nanobodies and the nb-IgE formats

Nanobodies were recloned into pET22b and expressed in Rosetta (DE3) cells according to the manufacturer's instructions. Thereby, a pelB signal sequence and a C-terminal his/V5 tag were added to the nanobodies allowing for secretion into periplasm and purification and detection.

Recombinant production of nb-IgE formats in mammalian HEK293 cells or FreeStyle 293-f was essentially performed using a vector system as described recently [2, 3]. Therefore, nanobodies were amplified using 5' and 3' primers. The PCR products were inserted between a human immunoglobulin-derived signal sequence and the IgE Fc domains.

Purification of nanobodies and nb-IgE formats from supernatant of bacteria and mammalian cells, respectively, was performed by IMAC using Ni NTA resin according to established protocols.

Analyses of immunoreactivity

Assessment of the Nb-IgEs was preformed using SDS-PAGE and immunoblotting. For immunoblotting, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Visualization of proteins was then performed with AP-conjugated goat anti-human IgE antibody (Sigma Aldrich) and nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indoylphosphate.

For assessment of IgE reactivity in ELISA, microtiter plates (Greiner) were coated with

51 nApi m 1, rApi m 2 or rFcεRIa (2 μg/mL) in PBS at 4 °C and blocked with 40 mg/mL milk in TBS.
 52 Thereafter, nb-IgEs (1μg/ml) were incubated for 3 hours at room temperature. After washing,
 53 bound IgEs were detected using the same antibody as above and substrate solution (5 mg/mL 4-
 54 nitro-phenylphosphate, AppliChem).

55 The sIgE and total IgE antibody measurements were performed using immunoCAPs
 56 (Thermo Fischer Scientific) or using the Euroline assay DPA-Dx Insect venoms 2 (Euroimmun)
 57 according to the manufacturer's instructions.

60 **Supplementary data Figures**

62 **Supplementary data Figure 1: Generation of nanobodies against nApi m 1 and rApi m 2**

63 A: Structures of Api m 1 (1POC) and Api m 2 (1FCU). B: SDS-PAGE analysis under non-reducing
 64 conditions of native Api m 1 and recombinant Api m 2 produced in insect cells. C: ELISA analysis
 65 of lama serum IgG immunoreactivity to allergens used for immunization. Detection was performed
 66 with an anti-llama IgG antibody. Data are single measurements.

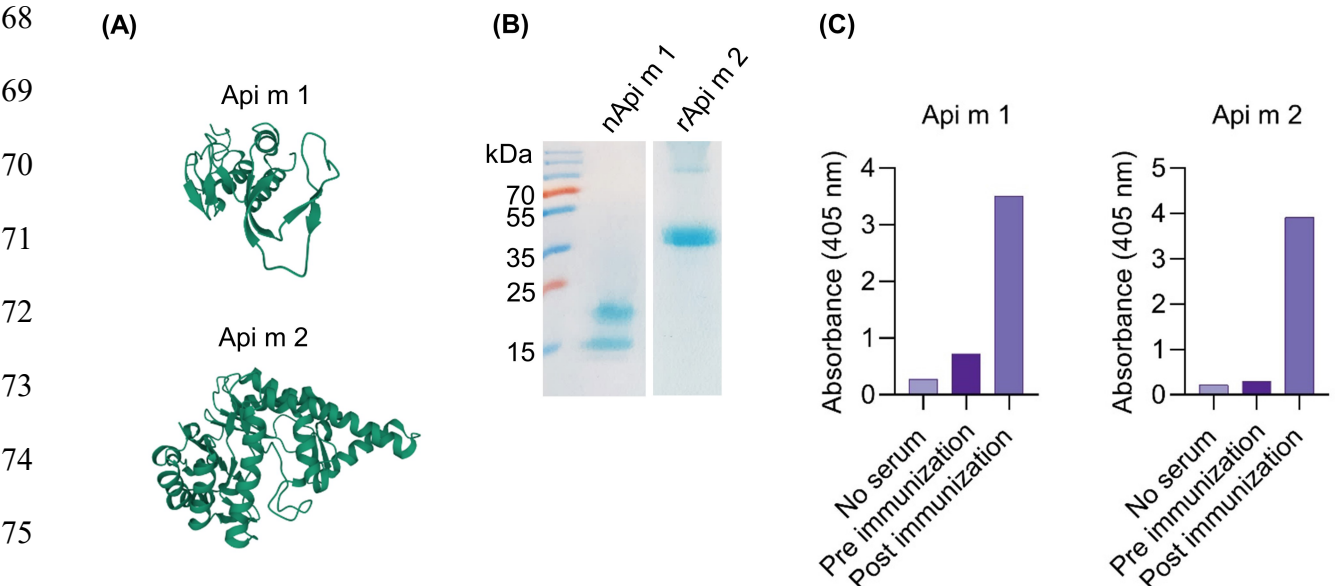


Figure S1_Aagaard et al.

Supplementary data Figure 2: Selection of the nanobody library

A and B: The enrichment of the library was assessed in ELISA by monitoring the immunoreactivity of polyclonal recombinant phages. Binding of phages to the immobilized antigen was detected using anti-M13 antibody conjugated to horseradish peroxidase. C and D: Immunoreactivity of monoclonal phages to the antigen used for selection was analyzed in ELISA as above. Data are single measurements.

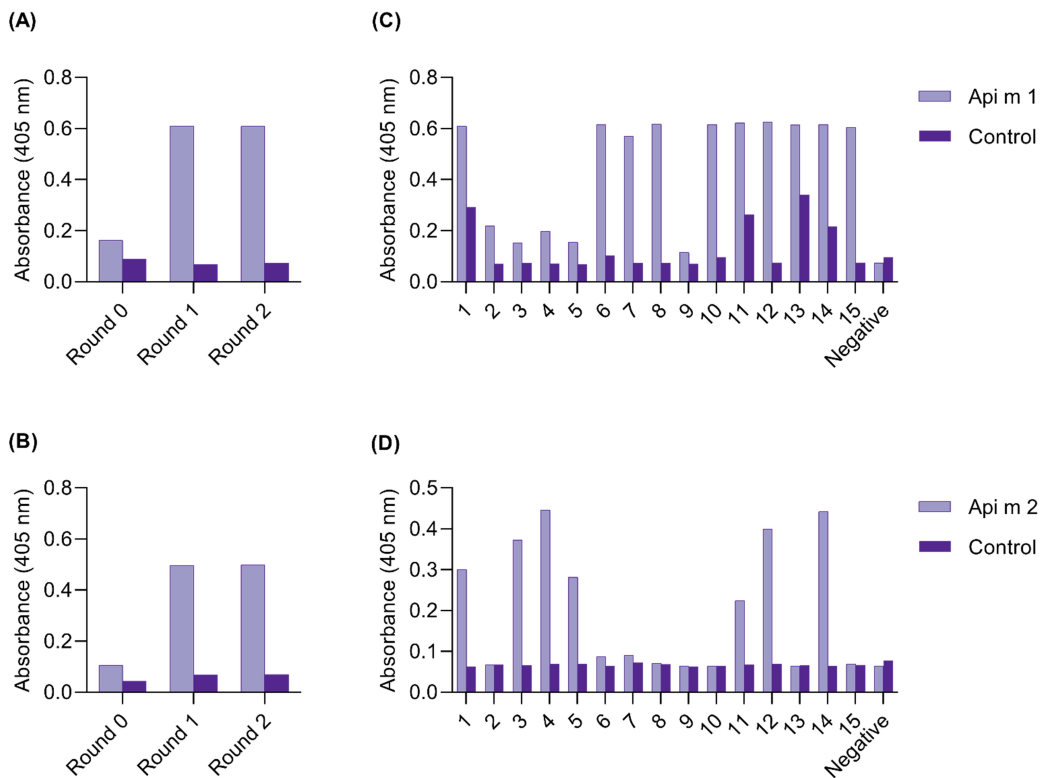


Figure S2_Aagaard et al.

Supplementary data Figure 3: Characterization of nanobodies expressed in bacteria

A: Recombinantly produced nanobodies were purified and assessed by SDS-PAGE, as exemplary shown for the AM1-1. Thereafter, the immunoreactivity of the purified nanobodies was verified in ELISA (B and C). B: Binding to the immobilized Api m 1 was detected in ELISA using an anti-V5 tag antibody and AP-conjugated goat anti-rabbit IgG. Data are mean $\pm$ s.d. of duplicates. C: Binding to the immobilized Api m 2 was detected in ELISA using a mouse anti-penta-his antibody and AP-conjugated rabbit anti-mouse IgG. Data are single measurements.

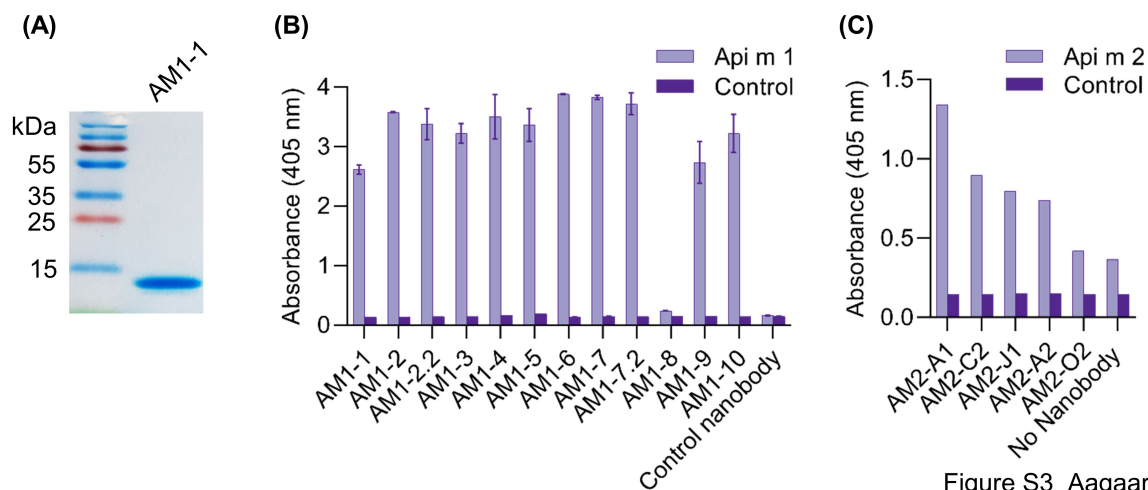


Figure S3_Aagaard et al.

Supplementary data Figure 4: Purification of nb-IgE from culture supernatant:

SDS-PAGE (left) and immunoblotting (right) analysis of the exemplary purification of the nb-IgE AM1-1 under non-reducing conditions. In the immunoblotting, the nb-IgE was detected using an anti-IgE antibody conjugated to alkaline phosphatase.

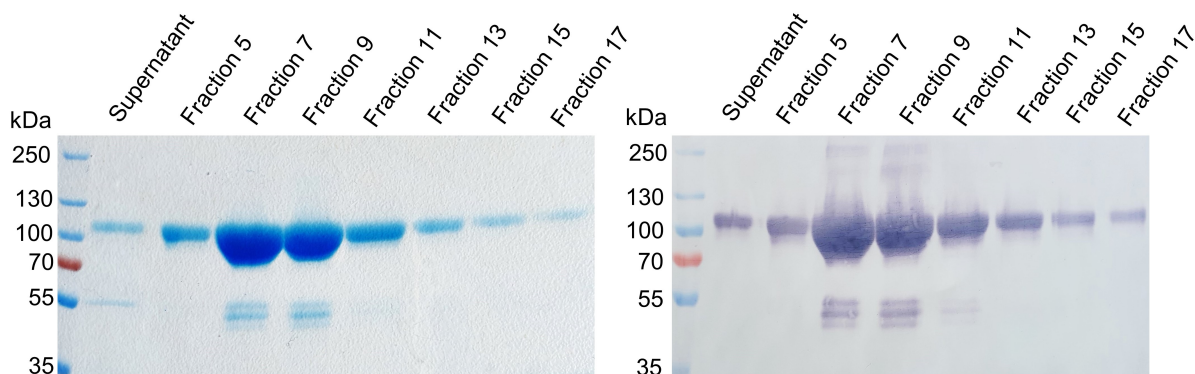


Figure S4_Aagaard et al.

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