Vaccine against peanut allergy based on engineered virus-like particles displaying single major peanut allergens

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Background: Peanut allergy is a severe and increasingly frequent disease with high medical, psychosocial, and economic burden for affected patients and wider society. A causal, safe, and effective therapy is not yet available.

Objective: We sought to develop an immunogenic, protective, and nonreactogenic vaccine candidate against peanut allergy based on virus-like particles (VLPs) coupled to single peanut allergens.

Methods: To generate vaccine candidates, extracts of roasted peanut (Ara R) or the single allergens Ara h 1 or Ara h 2 were coupled to immunologically optimized Cucumber Mosaic Virus–derived VLPs (CuMVtt). BALB/c mice were sensitized intraperitoneally with peanut extract absorbed to alum. Immunotherapy consisted of a single subcutaneous injection of CuMVtt coupled to Ara R, Ara h 1, or Ara h 2.

Results: The vaccines CuMVtt-Ara R, CuMVtt-Ara h 1, and CuMVtt-Ara h 2 protected peanut-sensitized mice against anaphylaxis after intravenous challenge with the whole peanut extract. Vaccines did not cause allergic reactions in sensitized mice. CuMVtt-Ara h 1 was able to induce specific IgG antibodies, diminished local reactions after skin prick tests, and reduced the infiltration of the gastrointestinal tract by eosinophils and mast cells after oral challenge with peanut. The ability of CuMVtt-Ara h 1 to protect against challenge with the whole extract was mediated by IgG, as shown via passive IgG transfer. FcγRIIb was required for protection, indicating that immune complexes with single allergens were able to block the allergic response against the whole extract, consisting of a complex allergen mixture.

Conclusions: Our data suggest that vaccination using single peanut allergens displayed on CuMVtt may represent a novel therapy against peanut allergy with a favorable safety profile. (J Allergy Clin Immunol 2019;■■■:■■■-■■■.)

Key words: *Food allergy, novel therapy, virus-like particles*

Peanut allergy (PA) is a severe disease and is a leading cause of anaphylactic reactions among food allergies. The prevalence of PA in Western countries ranges between 1.4% and 3% in children and is increasing.[1] The disease typically develops early in life and only in about 20% of cases an outgrow of the allergy is observed.[2] The economic and psychosocial consequences of PA are important.[3] Strict avoidance of peanut is the most common strategy used by patients with allergy, but is difficult to achieve. In terms of prevention, a randomized controlled trial in infants at high risk to develop PA found that early peanut consumption reduced the risk in comparison to peanut avoidance.[4] In contrast, another randomized controlled clinical trial in normal-risk infants found that early peanut exposure had no significantly protective effect on the development of PA.[5] Finally, an observational trial in newborns again indicated that early peanut exposure had a protective effect.[6]

In terms of therapy, several immunotherapy trials with peanut-allergic patients have been performed, mainly using oral, sublingual, and epicutaneous immunotherapy.[7-9] These therapies showed beneficial effects on PA, but were associated with a long phase of desensitization.[10] In addition, potentially dangerous systemic allergic reactions and disturbing gastrointestinal symptoms have been observed.[11,12] A recently published phase III clinical trial[13] investigating oral immunotherapy showed positive results relating to desensitization against peanut, but patients with a history of severe anaphylaxis with bronchial asthma and chronic gastrointestinal symptoms were excluded in advance, similarly to previous trials.[14] Thus, there is currently no causal, safe, and ideally effective therapy of PA currently available, especially for those patients with severe allergy.

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Abbreviations used
APC: Allophycocyanin
Ara R: Roasted peanut extract
CP: Coat protein
CuMVtt: Cucumber Mosaic Virus including tetanus toxin epitopes
FITC: Fluorescein isothiocyanate
IV: Intravenous/intravenously
MCPT-1: Mast cell protease-1
PA: Peanut allergy
PE: Phycoerythrin
SC: Subcutaneous/subcutaneously
VLP: Virus-like particle

Peanuts contain a mixture of 12 allergens and numerous isoforms; considered as major allergens are Ara h 1 and Ara h 3 (members of the cupin superfamily) as well as Ara h 2 and Ara h 6 (members of the prolamin superfamily)[15] although IgE specificities vary among peanut-allergic patients.

Most children with detectable peanut-specific IgE are not allergic to peanut.[16] A previous study identified the central role of peanut specific IgG_4 for clinical tolerance in sensitized but not allergic patients.[17] The IgG_4/IgE ratio to peanut was significantly greater in peanut-sensitized but tolerant patients compared with that seen in allergic subjects, indicating that excess of IgG_4 could contribute to clinical tolerance. Direct competition of IgG_4 with IgE for the allergen and binding of IgG_4-allergen complexes to the inhibitory receptor FcγRIIb on mast cells and basophils are supposed to be responsible for protection. These findings were consistent with observations in patients after successful peanut immunotherapy. These patients show an increase in specific IgG_4 levels most notably to the major allergen Ara h 2, suggesting that Ara h 2 is an important allergenic protein in peanut.[18]

Whether allergen immunotherapy works via rebalancing the T-cell response or via induction of antibodies is a long-standing debate.[19] A protective effect against cat allergy through direct administration of 2 Fel d 1–specific IgG_4 mAbs has been demonstrated recently in a clinical study with cat-allergic patients.[20] IgG_4 antibodies are thought to compete with IgE for Fel d 1 binding, thereby inhibiting the crosslinking of the FcεRI on mast cells and basophils. In addition, allergen-IgG immune complexes engage the inhibitory FcγRIIb, thereby blocking cellular activation. On the basis of these considerations, we postulate that a vaccine against peanut allergens able to induce a strong and specific IgG response may have the potential to protect peanut-allergic patients.

Virus-like particles (VLPs) are platforms for induction of protective antibodies, and several VLP-based vaccines are commercially available including against human papilloma virus and hepatitis B virus.[21] In a previous study, a vaccine consisting of Qβ-derived VLPs coupled to the cat allergen Fel d 1 has been shown to be highly immunogenic and able to induce specific IgG antibodies in mice. Immunization of Fel d 1–sensitized mice with Qβ-Fel d 1 protected against anaphylaxis after challenge with Fel d 1 allergen.[22] In a recent study, it has been shown that allergens displayed on Qβ-VLP are immunogenic but not reactogenic and fail to activate human mast cells.[23] VLPs could therefore constitute a platform to deliver allergens to peanut-allergic patients in in a non-reactogenic manner, while maintening a robust immunogenic effect.

In the current study, we aimed to develop and test vaccine candidates against PA on the basis of immunologically optimized VLPs derived from Cucumber Mosaic Virus including tetanus toxin epitopes (CuMVtt).[24] In addition to the immunogenic properties of other VLPs including the repetitive 3-dimensional scaffold (B-cell activation)[25] and the RNA content (stimulation of Toll-like receptor 7 and Toll-like receptor 8), CuMVtt contains the universal T-cell epitope derived from the tetanus toxin genetically fused into the structure. The preexisting T-cell memory for tetanus toxin is near universal in humans, and incorporation of the tetanus toxin epitope boosted T-cell response in randomly selected primary human T cells.[24]

Here, we tested the efficacy of vaccine candidates composed of CuMVtt coupled to the whole extract of roasted peanut or to the single major allergens Ara h 1 or Ara h 2 in a mouse model of PA and demonstrate strong immunogenicity and ability to protect against local and systemic allergic reactions to allergen extracts. We finally delineate the mechanism of action of the vaccines.

METHODS

Production of peanut extract, Ara h 1, Ara h 2

The extract of roasted peanut (Ara R) was obtained according to the protocol from Koppelman et al.[26] Briefly, 10 g of roasted peanut kernels (roasted salted peanuts [Felix] produced by Intersnack, Slominiki, Poland) were manually ground with mortar and pestle in 20 mM Tris-HCl and 2 mM EDTA buffer (pH 8.2), and the insoluble lipid part of peanut was removed through 3 low-speed centrifugations (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org).

Ara h 1 enrichment and purification from native peanut extract was performed with ammonium sulfate fractionations, anion-exchange chromatography, and size-exclusion chromatography (see Fig E1, *B* and *C*).

Recombinant peanut antigen Ara h 2 was produced in *Escherichia coli* C2566 cells transformed with pET42-Ara-h202-nhk. The biomass was sonicated in 10 mL buffer (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.1 mM PMSF, 0.1% TX-100, DNAse I [30 μg/10 mL], RNAse [0.5 mg/10 mL]). Sonication of the cells was performed for 16 minutes (0.5 interval/0.7 power, Hielscher UP200S). Additional 10 mL of the buffer was added and the suspension was mixed on a rotating mixer (10 rpm at room temperature for 1 hour). Inclusion bodies were collected by centrifugation at 11,000 rpm for 30 minutes and washed with the same buffer. Next, insoluble Arah202-nhk pellets were solubilized in 10 mL of 6 mol/L guanidine-HCl containing 100 mM NaCl, 5 mM EDTA, 10 mM dithiothreitol, and 20 mM Tris 8.5 and incubated at room temperature overnight. Then, the sample was centrifuged (11,000 rpm, 30 minutes) and slowly added to 110 mL refolding buffer (0.1 mol/L 3-(cyclohexylamino)-1-propane sulfonic acid [CAPS], pH 9.5, 0.9 mol/L arginine, and 0.3 mM reduced and 0.03 mM oxidized glutathione). After overnight incubation at room temperature, the mixture was dialyzed against 200 volumes of buffer containing 20 mM Tris-HCl and 50 mM NaCl (ON, +4°C) using SpectraPor dialysis membrane (12-14 kDa). Refolded Arah202-nhk was diluted with 50 mM Tris-HCL (pH 8.0), loaded onto Sepharose Q HP (XK16/20 column), and eluted with 1 mol/L NaCl in 50 mM Tris-HCl (pH 8.0). Arah202-nhk– containing fractions were finally purified using Superdex 200 column (see Fig E1, *D*).

Ara h 1 and Ara h 2 were identified by anti–Ara h 1 and anti–Ara h 2 polyclonal antibodies (Indoor Biotechnologies, Charlottesville, Va) on Western blots followed by incubation with horseradish peroxidase–conjugated secondary anti-rabbit IgG (Sigma-Aldrich, St Louis, Mo) (see Fig E1, *B*).

Production of CuMVtt

The production of CuMVtt was described in detail in Zeltins et al.[24] Briefly, RNA from CuVMtt-infected lily leaves was isolated using TRI reagent (Sigma-Aldrich). For cDNA synthesis, OneStep RT-PCR kit (Qiagen, Venlo,

The Netherlands) was used. The corresponding PCR products were cloned into the pTZ57R/T vector (Fermentas, Vilnius, Lithuania). After sequencing, the cDNA of CuMV coat protein (CP) gene was subcloned into the NcoI/HindIII sites of the pET28a(+) expression vector (Novagen, San Diego, Calif), resulting in the expression plasmid pETCuMVWT. The tetanus toxoid epitope coding sequence was introduced in the *CuMVWT* gene, by 2-step PCR mutagenesis, resulting in expression vector pET-CuMVtt. For CuMVtt VLPs, *E coli* C2566 cells (New England Biolabs, Ipswich, Mass) were transformed with the CuMVtt CP gene–containing plasmid pETCuMVtt. The expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside. The resulting biomass was collected by low-speed centrifugation and was frozen at −20°C. After thawing on ice, the cells were suspended in the buffer containing 50 mM sodium citrate, 5 mM sodium borate, 5 mM EDTA, and 5 mM mercaptoethanol (pH 9.0, buffer A) and were disrupted by ultrasonic treatment. Insoluble proteins and cell debris were removed by centrifugation (13,000 rpm, 30 minutes at 5°C, JA-30.50Ti rotor, Beckman, Palo Alto, Calif). The soluble CuMVtt CP protein in clarified lysate was pelleted using saturated ammonium sulfate (1:1, vol/vol) overnight at 4°C. Soluble CuMV-CP–containing protein solution was separated from the cellular proteins by ultracentrifugation in a sucrose gradient (20%-60% sucrose; ultracentrifugation at 25,000 rpm for 6 hours at 5°C [SW28 rotor, Beckman]). After dialysis of Cucumber mosaic virus (CuMV)-containing gradient fractions, VLPs were concentrated using ultracentrifuge (TLA100.3 rotor, Beckman; at 72,000 rpm for 1 hour, +5°C) or by ultrafiltration using Amicon Ultra 15 (100 kDa; Merck-Millipore, Cork, Ireland).

Generation of the vaccine CuMVtt-Ara R, CuMVtt-Ara h 1, and CuMVtt-Ara h 2

The peanut extracts Ara h 1 or Ara h 2 were modified for the subsequent coupling to CuMVtt VLPs with SATA according to the protocol of the manufacturer (Thermo Fisher Scientific, Waltham, Mass). SATA reactions were performed for 30 minutes at 23°C using 3.6× molar excess of SATA for Ara R or 3.3× for Ara h 1 and 10× for Arah202-nhk. Unreacted SATA was removed by washing the proteins 4 times with 5 mM $NaHPO_4$, pH 7.5, and 2 mM EDTA using Amicon Ultra-0.5, 10 K filtration units (Merck-Millipore). Free sulfhydryl groups in modified proteins were generated by deacetylation with 0.5 mol/L hydroxylamine (Thermo Fisher Scientific) and incubation for 2 hours at 23°C. The modified peanut extract, Ara h 1 or Ara h 2, was conjugated to CuMVtt using the cross-linker Succinimidyl 6-(beta-maleimidopropionamido)hexanoate (SMPH) (Thermo Fisher Scientific, 10-molar excess, 60 minutes, 23°C). The coupling reactions were performed with 0.3× molar excess of SATA-modified Ara R, 0.3× SATA-Ara h 1, or equal molar amount of Arah202-nhk regarding the CuMVtt (shaking at 23°C for 3 hours at 1200 rpm on DSG Titertek; Flow Laboratories, Irvine, United Kingdom). Unreacted SMPH and peanut proteins were removed using Amicon-Ultra 0.5, 100K (Merck-Millipore, Burlington, Mass). VLP samples were centrifuged for 2 minutes at 14,000 rpm for measurement on ND-1000.

Because of crosslinking of subunits, derivatization by SMPH leads to the characteristic ladder of CuMVtt monomers, dimers, trimers, tetramers, and so forth. The primary coupling band for CuMVtt-Ara h 1 appears as 1 CuMVtt monomer linked to 1 Ara h 1 protein at approximately 110 kDA. Coupling efficiency was calculated by densitometry (as previously described for IL17A-CMVTT vaccine[24]), with a result of approximately 20% to 30%, meaning 60 peanut allergen molecules were linked to 1 particle.

Coupling confirmation and densitometry measurement were achieved on SDS-PAGE as shown in Fig 1, *A*.

Mice experiments

BALB/c mice (Envigo, Huntingdon, United Kingdom) were purchased at the age of 6 weeks and kept at the DKF animal facility (Murtenstrasse 31, Bern, Switzerland). All animals were used for experimentation according to protocols approved by the Swiss Federal Veterinary Office (licence no. BE 70/18).

To test the immunogenicity of the vaccines, 6-week-old naive BALB/c mice were immunized subcutaneously (SC) either with CuMVtt coupled to Ara h 1 (30 μg CtMVtt-Ara h 1) or with Ara h 1 (10 μg). IgG levels were measured 7 and 14 days after vaccination.

Six-week-old naive BALB/c mice were sensitized to peanut by injecting twice intraperitoneally with 5 μg Ara R mixed in 200 μL alum (10 mg/mL $Al(OH)_3$; Alhydrogel; InvivoGen, Toulouse, France). For efficacy experiments, sensitized mice were vaccinated once SC with 30 μg CuMVtt-Ara R, CuMVtt-Ara h 1, or CuMVtt-Ara h 2 in 200 μL PBS; 2 weeks after sensitization, control groups were injected with CuMVtt 30 μg. Challenge was performed intravenously (IV), via skin prick test or gavage.

For induction of anaphylaxis, sensitized mice were challenged IV with 20 μg Ara R in 200 μL PBS. Temperature was measured with a rectal probe thermometer (Vetronic Services Ltd, Devon, United Kingdom) before IV antigen challenge and monitored for 50 minutes after challenge. To assess physical fitness and activity after challenge, open field activity tests were performed starting 10 minutes after IV injection. Distance moved was recorded for 10 minutes for all groups and evaluated with the video tracking system EthoVision XT-11 (Noldus Information Technology, Wageningen, The Netherlands).

For passive vaccination, IgG antibodies were induced with CuMVtt-Ara h 1 immunization of naive 6-week-old BALB/c mice. Pooled serum was collected and IgG isolated through Protein G sepharose column (GE Healthcare, Chicago, Ill; according to manufacturer's instruction). Sensitized mice received once 150 μg of isolated IgGs in 200 μL PBS 24 hours before challenge, and the control group 200 μL PBS. To assess the role of the inhibitory FcγRIIb receptor in basophils and mast cells on a systemic level, mice were injected IV with 150 μg anti-FcγRIIb antibodies (provided by M. Cragg, Antibody and Vaccine Group, Southampton, United Kingdom) 24 hours before the IV challenge with peanut extract. At the same time, as a control isotype antibody, 150 μg anti-Histidin antibodies were injected in CuMVtt-Ara h 1–vaccinated mice.

The local allergic reaction was assessed by ear prick test. Mice were injected IV with 200 mL of Evans blue solution (0.5% in PBS). Afterward, a drop of peanut extract solution (180 μg/20 μL PBS) was placed onto the outer ear skin of anesthetized mice. Pricks on the ear skin were performed with 23G (0.6 mm × 25 mm) needles (Microlance; Becton Dickinson, Allschwill, Switzerland). To assess FcγRIIb receptor function, designed ankyrin repeat proteins[27] against mouse FcγRIIb receptor were used for blocking the receptor by means of local SC injection on the ears 10 minutes before the ear prick. Dye extravasations started immediately after antigen challenge. Forty minutes later, mice were sacrificed and ears were collected. Ears were collected, photographed with C300 device (Azure Biosystems, Dublin, Calif), and surface of the blue extravasation was quantified by Fiji ImageJ software.

The infiltration of eosinophils in the gastrointestinal tract was assessed as follows: Sensitized and vaccinated mice were challenged for 3 days, once per day, with 100 mg roasted peanut or PBS via gavage. Mice were then sacrificed, stomach and proximal jejunum were collected, washed in PBS, fixed in paraformaldehyde 4% for 4 hours, and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin-eosin. Five random fields per section (2 for stomach, 3 for jejunum) were examined with a Imager.M2 (Zeiss, Oberkochen, Germany) microscope and scored for infiltration of eosinophils in the lamina propria according to a morphologic scale (1, no infiltration of eosinophils; 2, moderate infiltration of eosinophils; 3, strong infiltration of eosinophils).

For flow-cytometry analysis and eosinophil quantification, the first 10 cm of the small intestine was collected and mesenteric tissue was removed. The small intestine was longitudinally opened, washed in Hanks' balanced salt solution, and cut into small pieces. Epithelial cells were removed through incubation for 20 minutes (37°C, on incubator shaker) in 35 mL prewarmed HBSS containing 2% horse serum 0.005 mol/L EDTA (Sigma-Aldrich) and 0.000308% dithiothreitol (Sigma-Aldrich). Then, intestinal pieces were washed in HBSS containing 2% horse serum and digested for 20 minutes in prewarmed HBSS containing 2% horse serum, collagenase IV (50 mg/100 mL [Sigma-Aldrich]), and DNase1 (2 mg/100 mL [Roche Diagnostics, Rotkreuz, Switzerland]) solution (37°C, on incubator shaker). The resulting suspension was filtered (40-μm pore) and centrifuged for 4 minutes at 370*g*. The pellet was collected in HBSS containing 2% horse serum and DNase1 (2 mg/100 mL [Roche Diagnostics]). For detection of eosinophils, cells were stained in HBSS on ice for 30 minutes: viability dye live/dead fixable blue dead cell stain

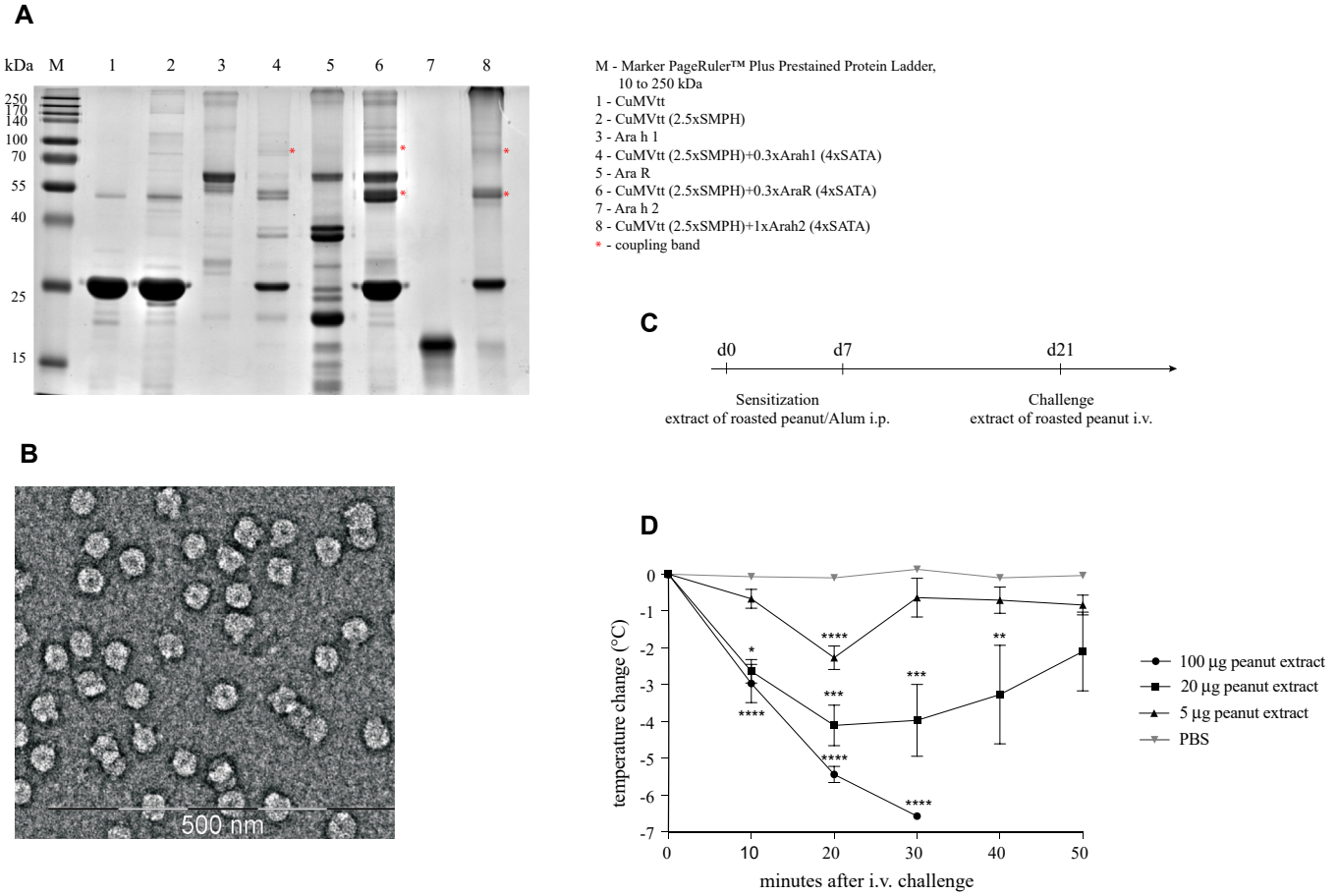

FIG 1. Vaccine generation and establishment of a mouse model of PA. **A,** Analysis of peanut extract, Ara h 1, Ara h 2, and coupling reactions with CuMVtt by SDS-PAGE 4%-12% gradient. Coupling bands show successful reaction. **B,** Electron microscopy image of CuMVtt coupled to Ara h 1. Vaccine particles are morphologically not aggregated (for dynamic light scattering analysis, see Fig E2). **C,** Experimental design for establishment of PA mouse model. Six-week-old naive BALB/c mice were injected i.p. with 5 μg Ara R mixed in 200 μL alum at day 0 and day 7. Challenge with Ara R was performed at day 21. **D,** Temperature after challenge was measured rectally every 10 minutes for 50 minutes. Dose-dependent anaphylaxis corresponding to temperature drop after challenge with Ara R; means ± SEM are shown (n = 3 mice per group). Data are representative of 2 independent experiments. Mice showing temperature less than 32°C were euthanized (challenge with 100 μg Ara R) according to regulatory protocols. Anaphylaxis curves were analyzed by repeated-measures 2-way ANOVA test, comparing the PBS-challenged group with peanut extract–challenged groups (dose-dependent anaphylaxis). *i.p.*, Intraperitoneally.

kit (Invitrogen, Carlsbad, Calif), CD45-BV711 (BioLegend, San Diego, Calif), CD11b-phycoerythrin (PE) (Becton Dickinson, Franklin Lakes, NJ), Siglec F-PerCpCy5.5 (Becton Dickinson), GR1-AlexaF700 (BioLegend). Intestinal mast cells were stained with PE-Cy7-CD45 (BioLegend), allophycocyanin (APC)-c-Kit (Becton Dickinson), and fluorescein-5-isothiocyanate (FITC)-FcεRI (Thermo Fisher Scientific). Measurements were performed with FACS LSR II (Becton Dickinson) cytometer and analysis with FlowJo software (FlowJo LCC, Becton Dickinson).

ELISA for determining peanut specific IgG

Ninety-six-well Nunc Maxisorp ELISA plates (Thermo Fisher Scientific) were coated with 1 μg/mL in carbonate buffer at 4°C overnight. After blocking with PBS/0.15% casein solution for 2 hours, plates were washed 5 times with PBS/0.05% Tween. Serial dilutions of sera were added to the plates and incubated for 2 hours at 4°C. Plates were then washed 5 times with PBS/0.05% Tween. Thereafter, horseradish peroxidase–labeled goat anti-mouse IgG (The Jackson Laboratory, Bar Harbor, Me) antibodies were incubated at 4°C for 1 hour. For determination of peanut extract specific IgG subclasses, biotin-labeled mouse anti-mouse IgG_1 (The Jackson Laboratory), biotin-labeled mouse anti-mouse IgG_{2a} (Becton Dickinson), or biotin-labeled rat anti-mouse IgG_{2b} (BioLegend) were used as detection antibodies for 1 hour at 4°C. Thereafter, horseradish peroxidase–labeled streptavidin (DakoCytomation, Copenhagen, Denmark) was incubated at 4°C for 1 hour. ELISAs were developed with 3,30,5,50-tetramethyl-benzidine and H_2O_2 and stopped with 1 mol/L sulfuric acid. ODs were measured at 450 nm. Half-maximal antibody titers are defined as the reciprocal of the dilution leading to half of the OD measured at saturation.

ELISA for determining mast cell protease-1

Mast cell protease-1 (MCPT-1) levels were measured in serum of mice collected 1 hour after IV challenge. The experiments were performed according to the manufacturer's instructions (MCPT-1 Mouse uncoated ELISA Kit, Invitrogen, Thermo Fisher).

In vivo reactogenicity of CuMVtt vaccine

Sensitized mice were challenged IV 2 weeks after completed sensitization with Ara R 20 μg or CuMVtt-Ara R 60 μg (corresponding dose of free and CuMVtt-bound allergen). Anaphylaxis was assessed by measuring the

temperature every 10 minutes for 50 minutes. To assess local reactogenicity of single major allergens, sensitized mice were challenged with Ara h 1 or CuMVtt-Ara h 1, in parallel with Ara h 2 and CuMVtt-Ara h 2 (with an allergen concentration of 0.3 mg/mL).

In vitro reactogenicity of CuMVtt vaccine, basophil activation assay

Experiments with blood of peanut-allergic patients were approved by the local ethics committee (KEK-Number 2018-00204). The experiments were performed according to the manufacturer's instruction (Flow CAST, Bühlmann, Switzerland). Briefly, whole blood of peanut-allergic patients was incubated with free Ara h 2 or Ara h 2 bound to CuMVtt (same concentration related to the contained allergen). Basophils were detected with PE-fluorescence– labeled anti-CCR3 mAb. Cell activation was determined by FITC-fluorescence–labeled anti-CD63 mAb. Measurements were performed with FACS Canto (BD Biosciences, Allschwil, Switzerland) flow cytometer and analysis with Flowjow software (FlowJo LCC).

Simultaneous binding and colocalization of IgE and IgG on basophils

Whole blood cells from naive or peanut-sensitized mice were incubated after lysing of the erythrocytes (Lysing buffer; Lonza, Walkersville, Md) with serum of naive or CuMVtt-Ara h 1–immunized mice (1:5) together with peanut extract (1 μg/mL) in RPMI 164 for 30 minutes at room temperature. After washing, cells were stained with anti-mouse IgE-FITC (BD Becton Dickinson, Allschwil, Switzerland), anti-mouse CD49b-APC (BioLegend), and anti-mouse IgG-PE (Jackson ImmunoResearch, Cambridgeshire, United Kingdom). Measurements were performed with FACS Canto (BD Biosciences) and analysis with FlowJo software (FlowJo LCC).

Imaging flow cytometry and analysis using Amnis IDEAS software

Imaging flow cytometry was performed using Image Streamx flow cytometer and the compatible INSPIRE system software (Amnis Corporation, Seattle, Wash). Cells were measured at 40× magnification and a flow speed flow coefficient below 0.2% indicating a stable core stream. Single cells were gated on the basis of "area" and "aspect ratio" features of the bright-field channel, which was set on channel 1. Focused cells were selected on the basis of "gradient root-mean-square" feature, which measures the resolution of an image, whereby values above 60 were considered for further analysis. Basophils were gated on the basis of APC anti-CD49b and FITC anti-IgE intensity. The data were analyzed using the IDEAS software (Amnis Corporation). IgE and IgG colocalization was assessed in IgG-positive basophils using "bright detail similarity" features of the fluorescence emitted by FITC anti-IgE and PE anti-IgG in the colocalization wizard provided by the IDEAS software.

In vitro inhibition of mast cell activation

Murine bone marrow–derived mast cells were cultured from wild-type BALB/c mice, as described in Stassen et al.[28] Cells were sensitized against peanut by incubation with serum derived from peanut-sensitized mice (ratio 1:10 in medium) overnight. After washing, mast cells were challenged with peanut extract (in a concentration of 1 μg/mL) preincubated with serum of naive mice or with serum of CuMVtt-Ara h 1–vaccinated mice (ratio 1:10 in medium) for 30 minutes in the incubator (37°C). After washing, cells were stained with anti–CD63-APC (BioLegend) to detect activation. Measurements were performed with FACS Canto (BD Biosciences) and analysis with FlowJo software (FlowJo LCC).

Statistical analysis

Statistical tests were performed with GraphPad Prism 6.0 (GraphPad Software, Inc, La Jolla, Calif). Statistical significance is displayed as $P \leq .05$ (*), $P \leq .01$ (**), $P \leq .001$ (***), and $P \leq .0001$ (****). Groups for IgG levels, dot surface after skin prick test, open field results (distance moved and velocity), and area under the curve were analyzed by unpaired 2-tailed Student *t* test. Anaphylaxis curves were analyzed by repeated-measures 2-way-ANOVA test.

RESULTS

Coupling of Ara R, Ara h 1, or recombinant Ara h 2 to CuMVtt

To generate and test different vaccine candidates against PA, we chemically coupled either the mixture of Ara h allergens contained in Ara R, or the purified single major allergen Ara h 1 or the recombinant Ara h 2 to the repetitive surface of CuMVtt, followed by removal of free allergen (see Figs E1 and E2 in this article's Online Repository at www.jacionline.org).[24] Details for the allergen coupling are shown by SDS-PAGE in Fig 1, *A*. Densitometric analysis shows a coupling efficiency of about 20% to 30%. Therefore, approximately 15 to 20 μg of allergens is contained per 60 μg of vaccine. Fig 1, *B*, shows with Ara h1 as an example that the structure of VLPs is preserved after coupling.

Vaccination with CuMVtt-Ara R, CuMVtt-Ara h 1, and CuMVtt-Ara h 2 protects against anaphylaxis

To establish a mouse model for PA, BALB/c mice were sensitized intraperitoneally at day 0 and day 7 with 5 μg Ara R absorbed to alum. For induction of anaphylaxis, sensitized mice were challenged IV with different doses of Ara R in 200 μL PBS. Rectal temperature was assessed at the time point of injection and every 10 minutes for 50 minutes after challenge (Fig 1, *C*). As shown in Fig 1, *D*, the dose-dependent temperature drop as a correlative parameter for anaphylaxis was assessed after allergen challenge. Mice receiving 100 μg peanut extract showed a temperature less than 32°C after 30 minutes and were euthanized.

To assess whether vaccinated animals were protected against anaphylaxis, BALB/c mice were vaccinated SC 2 weeks after sensitization with a single dose of 30 μg CuMVtt-Ara R, CuMVtt-Ara h 1, or CuMVtt-Ara h 2, or as a control CuMVtt alone (Fig 2, *A*). SC administration of CuMVtt alone or CuMVtt coupled to the allergens did not induce anaphylactic reactions in allergic mice, as shown by constant body temperature after injection (data not depicted). In contrast, SC injection with the corresponding amounts of free peanut allergens induced a significant anaphylactic reaction (see Fig E3 in this article's Online Repository at www.jacionline.org).

Two weeks after vaccination, all groups were challenged IV with 20 μg Ara R and body temperature was monitored for 50 minutes (Fig 2, *A*). Groups immunized with CuMVtt alone showed a severe drop in body temperature. In contrast, mice vaccinated with CuMVtt-Ara R, CuMVtt-Ara h 1, and CuMVtt-Ara h 2 were protected from anaphylactic reactions (Fig 2, *B*). An unexpected observation was the displayed protection against the whole extract when mice were vaccinated against single allergens (Ara h 1 and Ara h 2), which allowed us to pursue new lines of investigation to gain a better understanding and insights into the potential mechanism driving the protection induced by the vaccine.

To determine the role of mast cells and IgE in the observed anaphylaxis, serum levels of MCPT-1 were measured by ELISA in an exemplary way for mice immunized against Ara h 1. Fig 2, *C*, shows lower serum levels of MCPT-1 in the CuMVtt-Ara

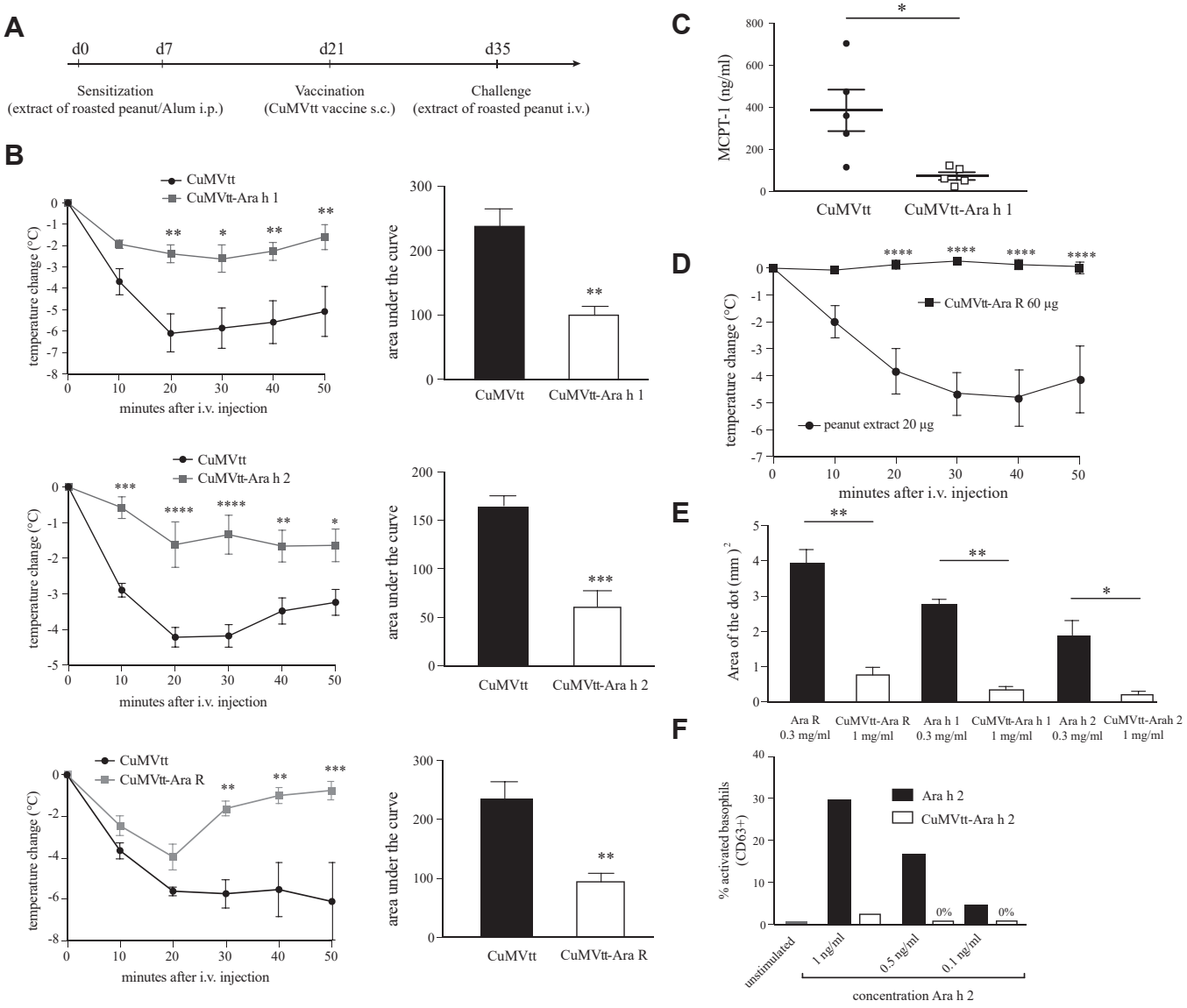

FIG 2. Vaccine CuMVtt-Ara h 1, CuMVtt-Ara h 2, and CuMVtt-Ara R protect against anaphylaxis in a mouse model of PA. **A,** To assess the efficacy of the generated vaccine, mice were sensitized with i.p. injection of 5 μg Ara R mixed in 200 μL alum at day 0 and day 7. Mice were vaccinated with 30 μg of CuMVtt-Ara h 1, CuMVtt-Ara h 2, or CuMVtt-Ara R on day 21. Challenge was performed on day 35 with 20 μg Ara R. **B,** Temperature after challenge to assess anaphylaxis was measured rectally every 10 minutes for 50 minutes. The *left panel* shows temperature course after challenge. Means ± SEM are shown (n = 4-5 mice per group). Data are representative of 3 independent experiments. Statistical significance was analyzed by 2-way-ANOVA test. The *right panel* shows statistical analysis performed with unpaired *t* test of related area under the curve (depicted ± SEM). **C,** To assess the role of mast cells after challenge in CuMVtt-vaccinated mice compared with CuMVtt-Ara h 1–vaccinated mice, serum MCPT-1 levels were measured in ELISA. Data are representative of 2 independent experiments (depicted ± SEM). **D,** Displaying allergens on CuMVtt reduces its reactogenicity. To show this, BALB/c mice were sensitized with peanut extract and 2 weeks later challenged IV with 20 μg peanut extract or 60 μg CuMVtt-Ara R (corresponding amount of allergen present in the challenge). **E,** To assess local reactogenicity, sensitized mice were challenged with Ara h 1 or CuMVtt-Ara h 1, in parallel with Ara h 2 and CuMVtt-Ara h 2 (with an allergen concentration of 0.3 mg/mL). Means ± SEM are shown (n = 3 mice per group); the graph shows results representative of 3 independent experiments. **F,** Ara h 2 was able to activate *in vitro* human basophils of peanut-allergic patients (n = 3) in a basophil activation test (activated basophils defined as $CCR3^+$, $CD63^+$ cells by flow cytometry), whereas Ara h 2 coupled to CuMVtt did not activate basophils compared with unstimulated cells. The experiment was performed once per patient; depicted results are representative for all 3 patients. *i.p.*, Intraperitoneally.

h 1–vaccinated group than in mice vaccinated with CuMVtt 1 hour after IV challenge. These data confirm the protective effect of the vaccine against anaphylaxis. Furthermore, increased serum MCPT-1 levels in the control group after challenge indicate mast cell degranulation and IgE-dependent induction of anaphylaxis in this mouse model (in line with O'Konek et al[29]).

Displaying peanut extract on CuMVtt strongly reduces its reactogenicity

Absence of reactogenicity of a vaccine against PA plays a fundamental role for clinical translation, because allergic reactions are feared and potentially dangerous. For this reason, severely allergic patients are usually excluded from clinical trials for immunotherapy. To address the question of reactogenicity, BALB/c mice were sensitized with peanut extract and 2 weeks later challenged with 20 μg peanut extract or 60 μg CuMVtt-Ara R (corresponding to an equivalent amount of allergen present in the challenge). Temperature drop was extensive in the group challenged with free allergen (peanut extract), whereas animals challenged with peanut extract coupled to CuMVtt did not show altered temperature (Fig 2, *D*). Local reactogenicity was also monitored using skin prick test. To visualize extravasation, mice were pretreated with Evans blue IV before the prick test. As shown in Fig 2, *E*, mice challenged with Ara h 1, Ara h 2, and Ara R developed a stronger allergic extravasation compared with mice challenged with CuMVtt-Ara h 1, CuMVtt-Ara h 2, and CuMVtt-Ara R, respectively (allergen concentration of 0.3 mg/mL).

Next, we performed *in vitro* basophil activation tests with whole blood of peanut-allergic donors. We compared the expression of CD63, a marker for basophil degranulation, after whole blood incubation with Ara h 2 or CuMVtt-Ara h 2 in an equivalent concentration to the free allergen (30 ng/mL). Fig 2, *F*, shows basophil activation upon free allergen stimulation; in contrast, incubation with Ara h 2 displayed on CuMVtt failed to activate basophils, showing CD63 levels comparable to unstimulated blood. Collectively, these results show that displaying peanut allergens on CuMVtt strongly reduces their reactogenicity. This observation is consistent with previous experiments performed with the cat allergen Fel d 1 in free form or coupled to Qβ-VLPs.[23]

Immunogenicity of CuMVtt-Ara h 1 in naive and sensitized mice

Because Ara h 1 could easily be purified in large amounts from peanut extracts, CuMVtt-Ara h 1 was the preferred candidate from a production point of view and we focused subsequent experiments on this allergen as an exemplary model to explore this observed protection using a single-allergen approach.

To this end, we addressed the immunogenicity of CuMVtt-Ara h 1 in a next step. Naive BALB/c mice were immunized SC with 10 μg of Ara h 1 either coupled to CuMVtt or in free form. Peanut extract–specific serum IgG level was measured 7 and 14 days after immunization. As seen in Fig 3, *A*, peanut specific IgG titers were induced by Ara h 1 coupled to CuMVtt, whereas strongly reduced titers were detected on injection of free Ara h 1. Next, we investigated the immunogenicity of CuMVtt-Ara h 1 in mice previously sensitized with peanut extract by measuring IgG-subclass titers at day 34 (1 day before challenge). As shown in Fig 3, *B*, vaccination with CuMVtt-Ara h 1 led to an increase in peanut extract–specific IgG_1, IgG_{2a}, and IgG_{2b} titers compared with basal levels of IgG subclasses in sensitized mice immunized with CuMVtt. These date indicate that immunization with CuMVtt vaccines has an impact on all IgG subclasses.

Passive vaccination with IgG generated with CuMVtt-Ara h 1 protects against anaphylaxis

To investigate the specific role of IgG antibodies in protection against anaphylaxis, BALB/c mice were injected with CuMVtt-Ara h 1 and IgG antibodies were purified from serum using protein A. Transfer of 150 μg of total IgG antibodies into peanut-sensitized mice protected from anaphylactic reactions. Protection was in a similar range as achieved by the vaccine itself, indicating that IgG antibodies were the major driver of vaccine efficacy (Fig 3, *C*).

CuMVtt-Ara h 1 improves physical fitness of mice after IV challenge

Allergic mice challenged IV with peanut extract develop typical signs of systemic allergy including erected hairs and immobility in addition to hypothermia. To quantify fitness, we measured physical activity after challenge in an open field experiment. Effects of vaccination with CuMVtt-Ara h 1 on distances moved were recorded for 10 minutes, starting 10 minutes after IV peanut extract challenge. As shown in Fig 4, *A*, vaccination with CuMVtt-Ara h 1 resulted in significantly higher levels of distances the mice moved after challenge compared with the CuMVtt group, a finding consistent with the protection against temperature drop.

CuMVtt-Ara h 1 diminishes local mast cell degranulation in skin prick test

To examine the effect of vaccination with CuMVtt-Ara h 1 on local allergic reactions, skin prick tests were performed in peanut-sensitized mice vaccinated with CuMVtt-Ara h 1 or CuMVtt as control. Allergen challenge by pricking into the ear skin with peanut extract induced vascular leakage in CuMVtt-vaccinated mice. In contrast, CuMVtt-Ara h 1–treated animals showed significantly smaller extravasation surface (Fig 4, *B*).

CuMVtt-Ara h 1 reduces infiltration by eosinophils and mast cells in the intestinal tract after oral challenge

Next, we wanted to address the protective capacity of the vaccine in a model of chronic food allergy. To this end, we measured the local inflammation in the gastrointestinal tract after gavage of peanut-sensitized mice with ground peanut kernels in PBS. We quantified the infiltration of eosinophils in the lamina propria of stomach and proximal jejunum after oral challenge for 3 days. Five random fields per section (2 for stomach, 3 for jejunum) were examined by microscopy and scored for infiltration of eosinophils in the lamina propria according to a morphologic scale (as described in Levy et al,[30] 1: no infiltration of eosinophils, 2: moderate infiltration of eosinophils, 3: strong infiltration of eosinophils). As shown in Fig 4, *C*, eosinophil infiltration in the hematoxylin-eosin staining is reduced in mice immunized with CuMVtt Ara h 1 compared with mice treated with CuMVtt alone.

For quantification of eosinophil and mast cell infiltration into the lamina propria of the proximal small bowel, flow cytometry analysis was carried out. To this end, 10 cm of the proximal jejunum was collected, digested, and single-cell suspensions were stained for cell surface markers. Eosinophils were defined as

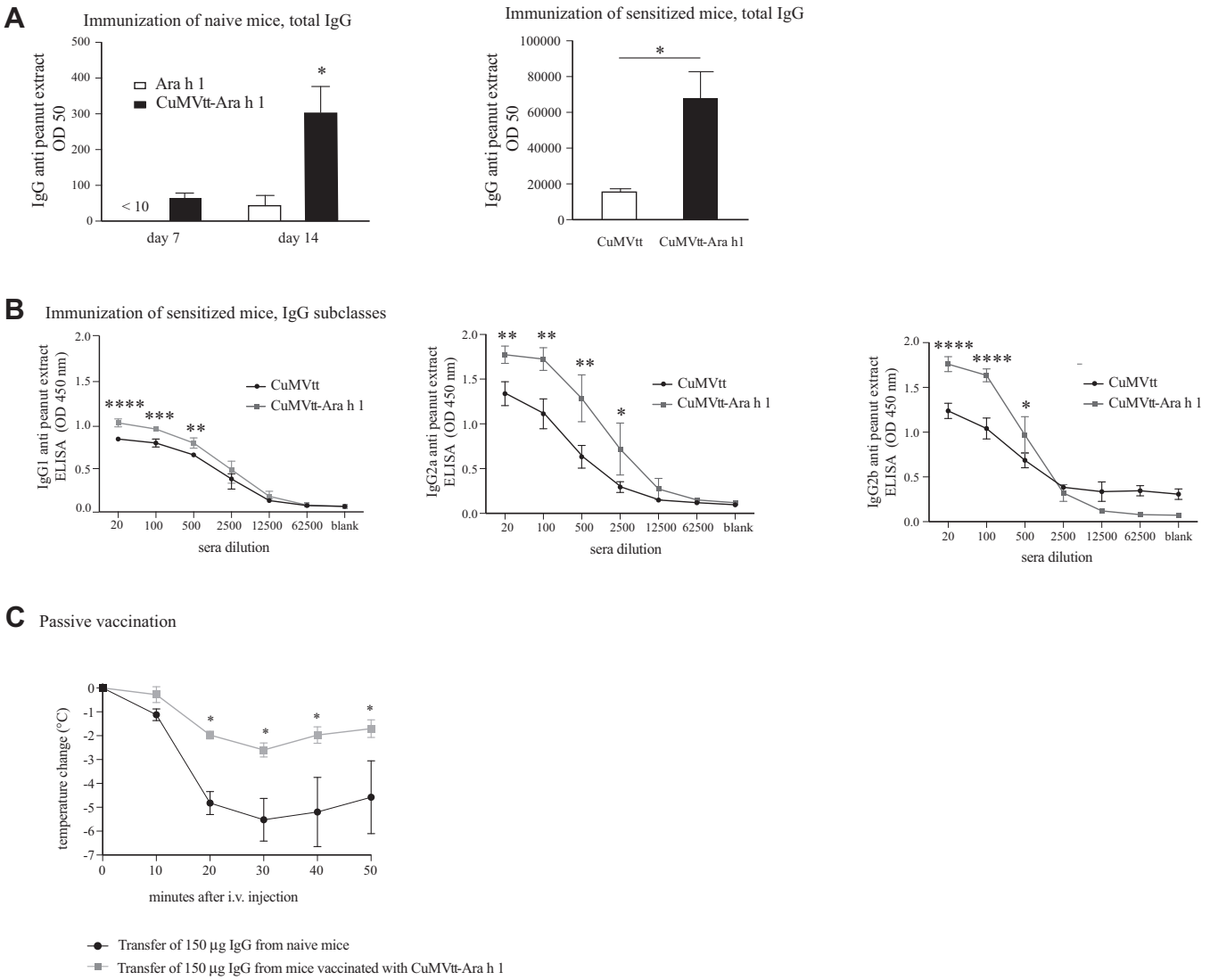

FIG 3. Immunogenicity of CuMV-Ara h 1. **A,** BALB/c mice were injected SC either with a total Ara h 1 amount of 10 μg in free form or coupled to CuMV (*left panel*). Serum anti–Ara R IgG levels were measured 7 days and 14 days postinjections. Shown are means ± SEM (n = 3 mice per group). Data are representative of 2 independent experiments. Peanut-sensitized mice were immunized with 30 μg CuMVtt or CuMV-Ara h 1 SC, and serum anti–Ara R total IgG levels were measured at day 34 (1 day before challenge) (*right panel*); shown are means ± SEM (n = 5 mice per group). Data are representative of 2 independent experiments. **B,** IgG-subclasses specific for Ara R were measured at day 34 (1 day before challenge); vaccination with CuMVtt-Ara h 1 led to an increase in OD50 titers of IgG_1, IgG_{2a}, and IgG_{2b}. Means ± SEM are shown (n = 4-5 mice per group). Data are representative of 3 independent experiments; statistical analysis was performed with multiple *t* test for corresponding dilutions. **C,** IgG antibodies were induced with CuMVtt-Ara h 1 immunization of naive 6-week-old BALB/c mice. Pooled serum was collected from naive or CuMVtt-Ara h 1–vaccinated mice and IgG antibodies were isolated through Protein G sepharose column. Sensitized mice received once 150 μg of isolated IgGs from vaccinated mice in 200 μL PBS IV 24 hours before challenge, and the control group 150 μg of isolated IgG from naive mice in 200 μL PBS IV. Challenge was performed with an IV injection of 20 μg Ara R. Means ± SEM are shown (n = 5 mice per group).

living $CD45^+$ $CD11b^+$ $SiglecF^{high}$ cells, and mast cells were defined as living $CD45^+$ c-Kit^+ $Fc\varepsilon RI^+$ (gating strategy in Fig E3). Fig 4, *D*, shows reduced eosinophil infiltration (left panel) and mast cell infiltration (right panel) in the CuMVtt-Ara h 1–vaccinated group, which reached levels found in PBS-challenged mice.

The inhibitory FcγRIIb receptor is required for protection induced by the single-allergen vaccine CuMVtt-Ara h 1

To investigate whether the inhibitory FcγRIIb receptor present on mast cells and basophils is involved in protection induced by IgG antibodies generated after CuMVtt-Ara h 1 vaccination,

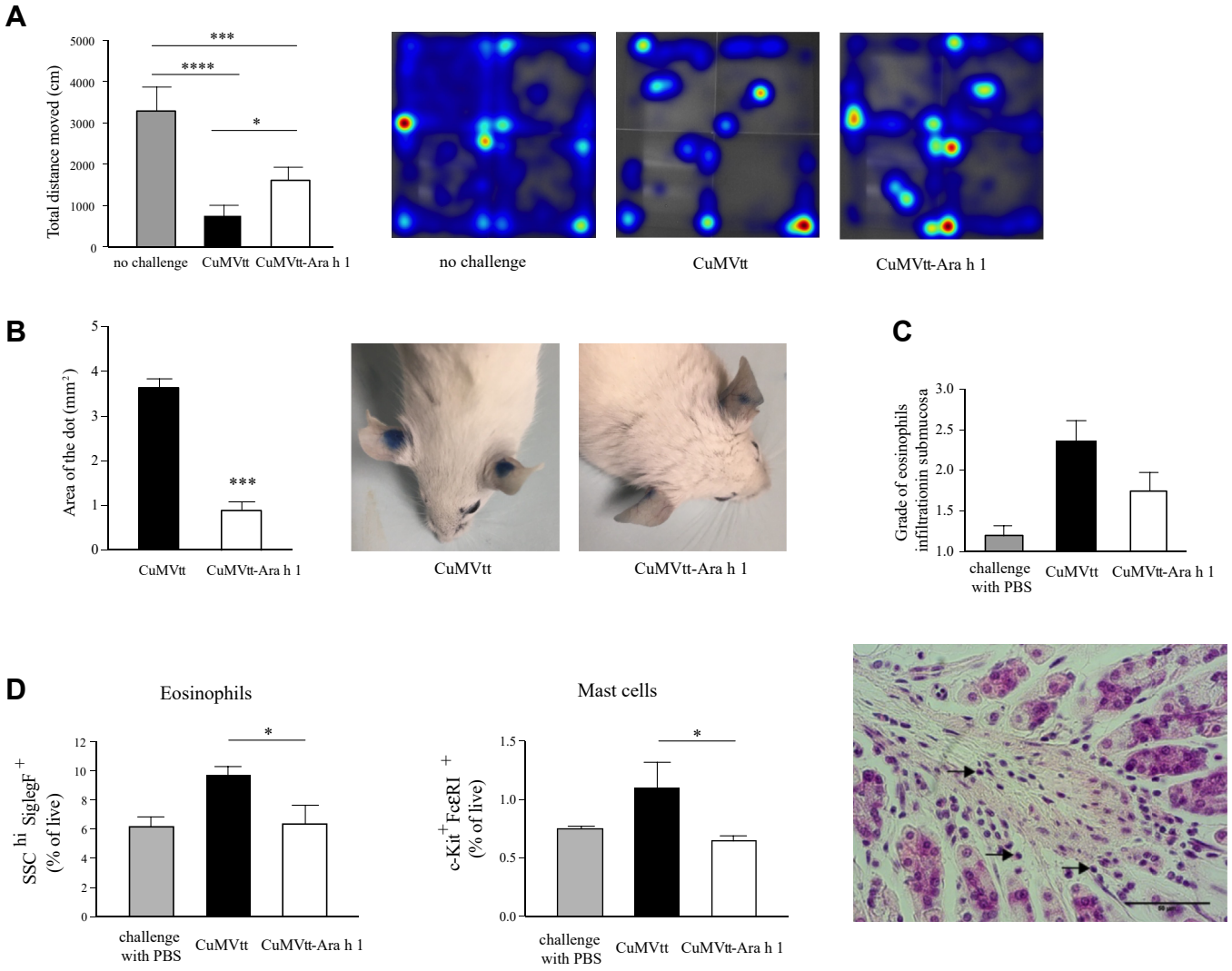

FIG 4. Effects of CuMVtt-Ara h 1 vaccine on physical fitness, skin prick test, and eosinophil infiltration in the proximal small bowel after challenge with Ara R in a mouse model of PA. **A,** Peanut-sensitized BALB/c mice were challenged IV with 20 μg Ara R, and total distance moved was measured starting 10 minutes after IV injection for 10 minutes in an open field experiment. CuMVtt-Ara h 1 increased the distance the mice moved after challenge. Means ± SEM are shown (n = 9 mice for no challenge group, n = 12 for CuMVtt and CuMVtt-Ara h 1 groups). Data are generated with 3 independent experiments. Heatmap plots show representative movements of 1 mouse during 10 minutes for each group. Statistical analysis of distance moved was performed with unpaired *t* test. **B,** Sensitized and vaccinated mice were injected IV with 200 mL of Evans blue solution, and prick test was performed with peanut extract on the ear skin under anesthesia. Surface of the extravasation was quantified using Fiji ImageJ software. Means ± SEM are shown (n = 3 mice per group). Data are representative of 2 independent experiments. Statistical significance was analyzed by unpaired *t* test. **C** and **D,** Sensitized and vaccinated mice were challenged for 3 days, once per day, with 100 mg roasted peanut or PBS via gavage. Mice were then sacrificed, and stomach and proximal jejunum were collected. Hematoxylin-eosin staining (Fig 4, *C*, arrows: example of eosinophil) and FACS analysis (Fig 4, *D*) show reduced eosinophil infiltration (*left panel*) and mast cell infiltration (*right panel*) in the lamina propria of CuMVtt-Ara h 1–vaccinated mice compared with the CuMVtt group. Means ± SEM are shown; data are representative of 2 independent experiments for hematoxylin-eosin sections (n = 5 mice per group). FACS analysis regarding the proximal jejunum shows results of 1 experiment (n = 5 per group for CuMVtt and CuMVtt-Ara h 1 groups; n = 3 for PBS challenge).

peanut-sensitized BALB/c mice were challenged IV 24 hours after injection of an anti-FcγRIIb mAb (AT 128) to block FcγRIIb receptor. As shown in Fig 5, *A*, protection conferred by vaccination with CuMVtt-Ara h 1 was abrogated by systemic injection of FcγRIIb-blocking antibodies. The protection was not affected when mice were injected with isotype control IgG.

The involvement of the inhibitory receptor FcγRIIb in protection was confirmed via skin prick test after locally blocking the FcγRIIb receptor with an inhibitor molecule (based on designed ankyrin repeat proteins technology) specific to FcγRIIb.[31] As shown above, CuMVtt-Ara h 1–vaccinated mice developed much smaller extravasation spots after ear skin prick test, but

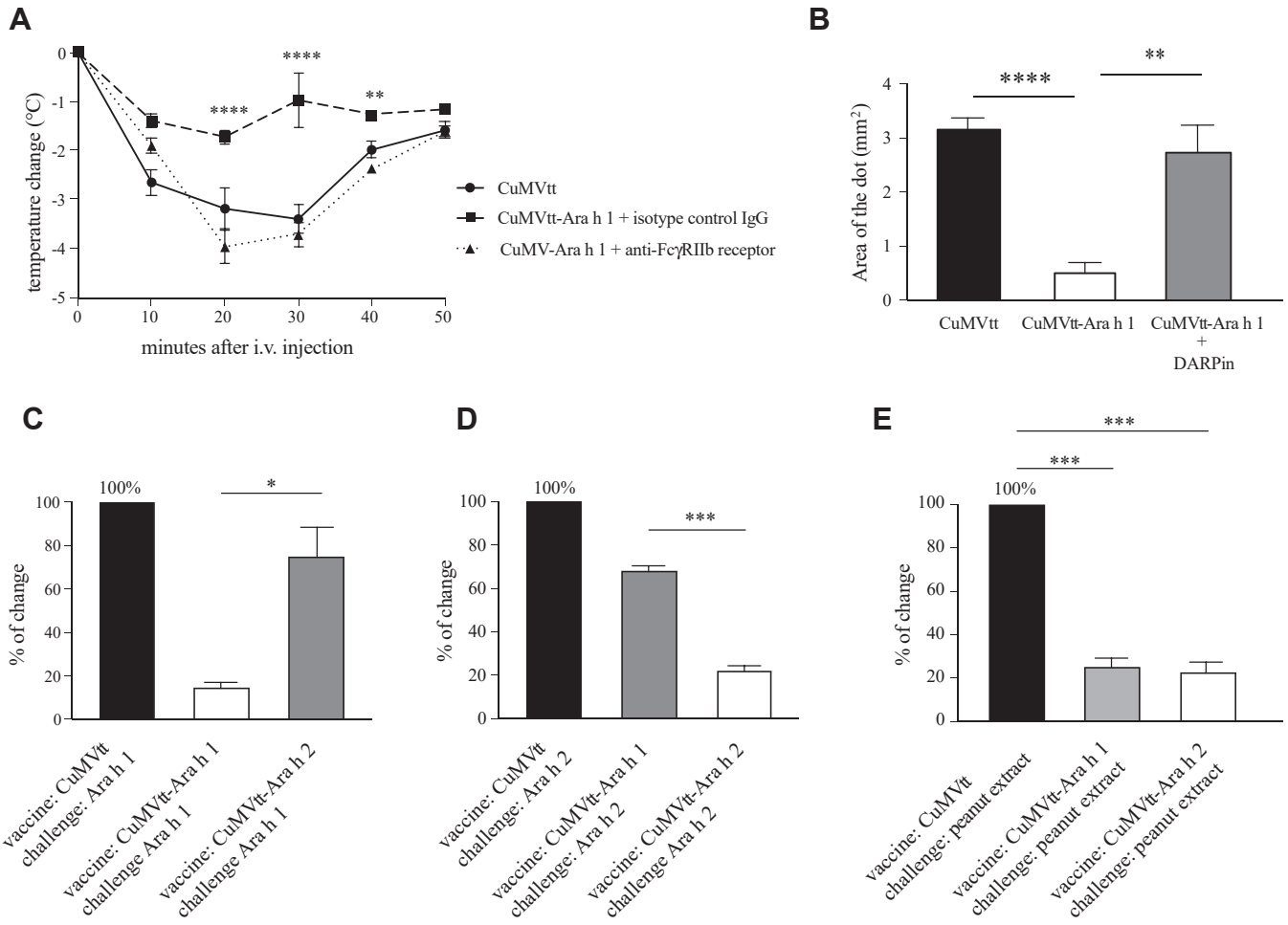

FIG 5. FcγRIIb is required for protection. **A,** To assess systemic FcγRIIb receptor function, 150 μg of anti-FcγRIIb mAb (AT 128) was administered IV 24 hours before allergen challenge; the control group received 150 μg of isotype IgG. Depicted statistical analysis shows difference between CuMVtt-Ara h 1 + anti-FcγRIIb and CuMVtt-Ara h 1 + isotype IgG control antibodies (n = 6 mice per group). **B,** To assess local FcγRIIb receptor function, a designed ankyrin repeat proteins molecule against FcγRIIb receptor was used for blocking the receptor by means of local SC injection on the ears 10 minutes before the ear prick. **C-E,** Protection induced by vaccination is specific for the displayed allergen on CuMVtt. Sensitized BALB/c mice were vaccinated against Ara h 1 or Ara h 2 and challenged in a skin prick test with the whole extract, Ara h 1, or Ara h2. Fig 5, *C*, Peanut-sensitized mice vaccinated with CuMVtt-Ara h 1 and challenged with Ara h 1 were protected. In contrast, vaccination with CuMV-tt-Ara h 2 failed to induce protection after challenge with Ara h 1. *D,* In parallel, vaccination with CuMVtt-Ara h 2 protected in case of challenge with Ara h 2, but vaccination with CuMV-Ara h 1 failed to protect mice after challenge with Ara h 2. *E,* CuMVtt-Ara h 1 and CuMV-Ara h 2 both protect in case of challenge with the whole peanut extract in peanut-sensitized mice. In *C-E*, the vaccine-induced protection is shown in % of change to the reference value obtained for the respective allergen used in the challenge and defined as 100%. Means ± SEM are shown (n = 3 mice per group). Data are representative of 2 independent experiments. Statistical analysis was performed with *t* test.

protection was abrogated by local injection of FcγRIIb-blocking designed ankyrin repeat proteins. Mice with blocked FcγRIIb show a comparable leakage to unvaccinated challenged mice, demonstrating that inhibitory receptor FcγRIIb is required for protection (Fig 5, *B*).

Protection induced by vaccination is specific for the displayed allergen on CuMVtt

The results so far indicate that vaccination against a single allergen protects against the whole extract in an FcγRIIb-dependent manner. This implies that immune complexes made of IgG and allergen are critical for protection. Hence, the vaccines should work only if the respective allergens are present in the challenge. To examine this question, we vaccinated peanut-sensitized mice with CuMVtt or CuMVtt-Ara h 1 or CuMVtt-Ara h 2 and challenged them in a skin prick test with the extract Ara h 1 or Ara h 2 (Fig 5, *C-E*). As expected, mice challenged with single allergens showed weaker reactions than the ones challenged with the whole extract (data not shown). We therefore normalized vaccine-induced protection to the reaction seen for the respective allergens used for challenge. As shown in Fig 5,

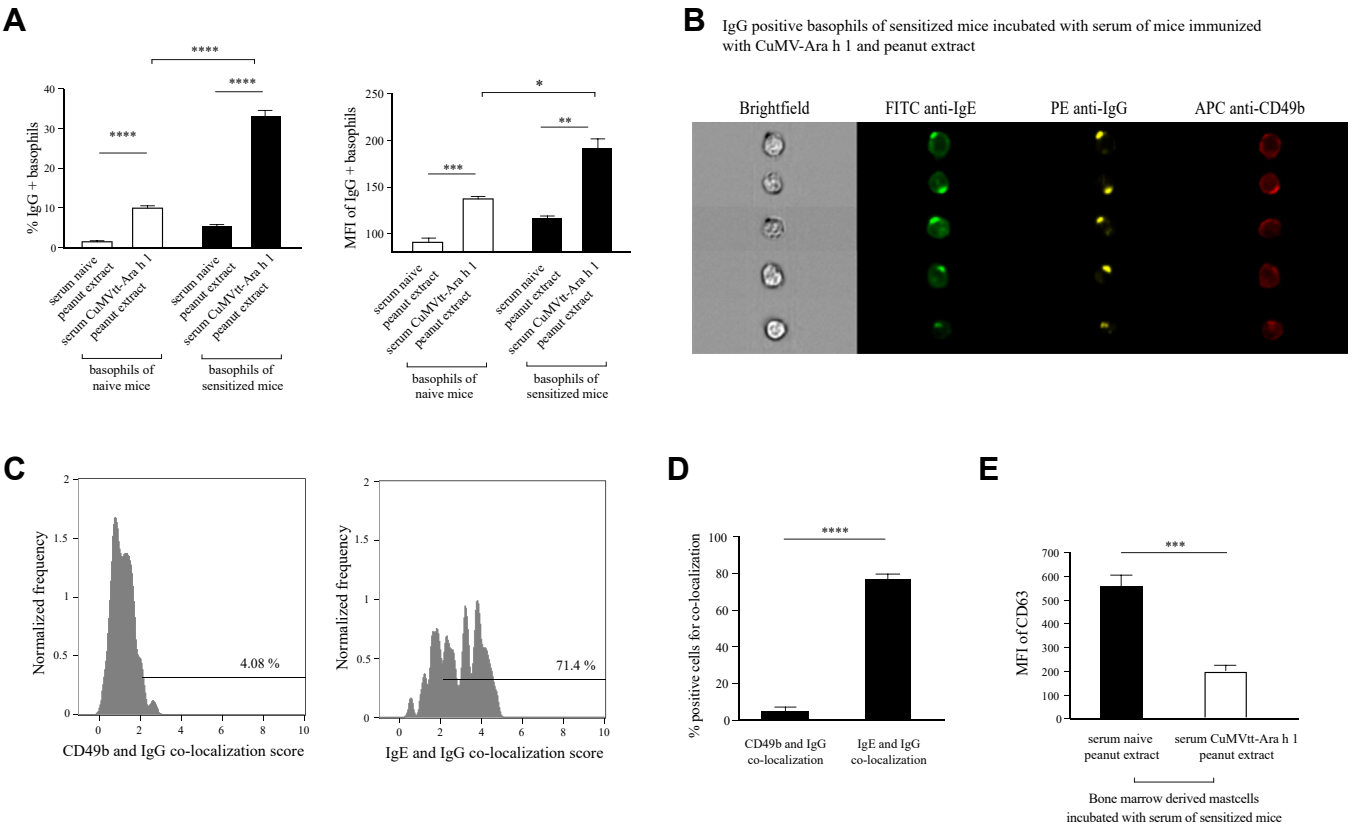

FIG 6. IgE and IgG antibodies bind to basophils simultaneously and show colocalization on the cell surface. **A,** To examine binding of IgE and IgG on basophils, whole blood cells from naive or peanut-sensitized mice were incubated with serum of naive or CuMVtt-Ara h 1– immunized mice together with peanut extract. IgG binding on basophils (CD49b and IgE-positive cells were gated) was quantified by flow cytometry (Fig 6, *A*) and imaging flow cytometry (**B-D**). Depicted data (Fig 6, *A*) were obtained in 3 independent experiments, ± SEM. Fig 6, *B-D,* Colocalization of IgE and IgG was assessed in IgG-positive basophils of sensitized mice incubated with serum of mice immunized with CuMVtt-Ara h 1 and peanut extract. Data ± SEM are shown (n = 3 mice per group). **E,** Murine bone marrow–derived mast cells were sensitized with serum of peanut-sensitized mice. Mast cells were challenged with peanut extract incubated with serum from naive or CuMVtt-Ara h 1–vaccinated mice. Activation of mast cells was quantified by flow cytometry gating CD63-positive cells. Data are representative for 3 independent experiments. *MFI*, Mean fluorescence intensity.

C and *D*, protection was observed only if mice were challenged with the allergen they were vaccinated against, but not with the other allergen. In contrast, mice challenged with the extract containing all allergens were protected as shown in Fig 5, *E*.

These results indicate that protection induced by immunization with single allergens is not based on cross-reactive antibodies but rather on the formation of immune complexes with the respective allergen used for immunization, causing engagement of the inhibitory FcγRIIb.

IgE and IgG antibodies bind to basophils simultaneously and show colocalization on the cell surface

A requisite for the hypothesized mechanism of protection through the inhibitory FcγRIIb receptor is the simultaneous binding of IgE and IgG-allergen complexes on basophils and mast cells. To examine this postulate, we incubated whole blood cells from naive or peanut-sensitized mice with serum of naive or CuMVtt-Ara h 1–immunized mice together with peanut extract and analyzed IgG binding on basophils (CD49b and IgE-positive cells were gated) by flow cytometry and imaging flow cytometry. As shown in Fig 6, *A*, basophils incubated with serum derived from CuMVtt-Ara h 1–immunized mice and with peanut extract bind significantly more IgG than do basophils incubated with serum of naive mice and with peanut extract. In other words, IgG antibodies induced by the vaccine CuMVtt-Ara h 1 bind on basophils in the presence of the allergen. This effect is significantly increased in basophils derived from peanut-sensitized mice compared with naive mice, indicating that the presence of peanut-specific IgE on the cell surfaces increases binding of IgG-peanut extract complexes. Colocalization of IgE and IgG was highly significant, whereas there was no colocalization of IgG or IgE with CD49b, which is a basophil surface marker not related to antibody binding (Fig 6, *C* and *D*).

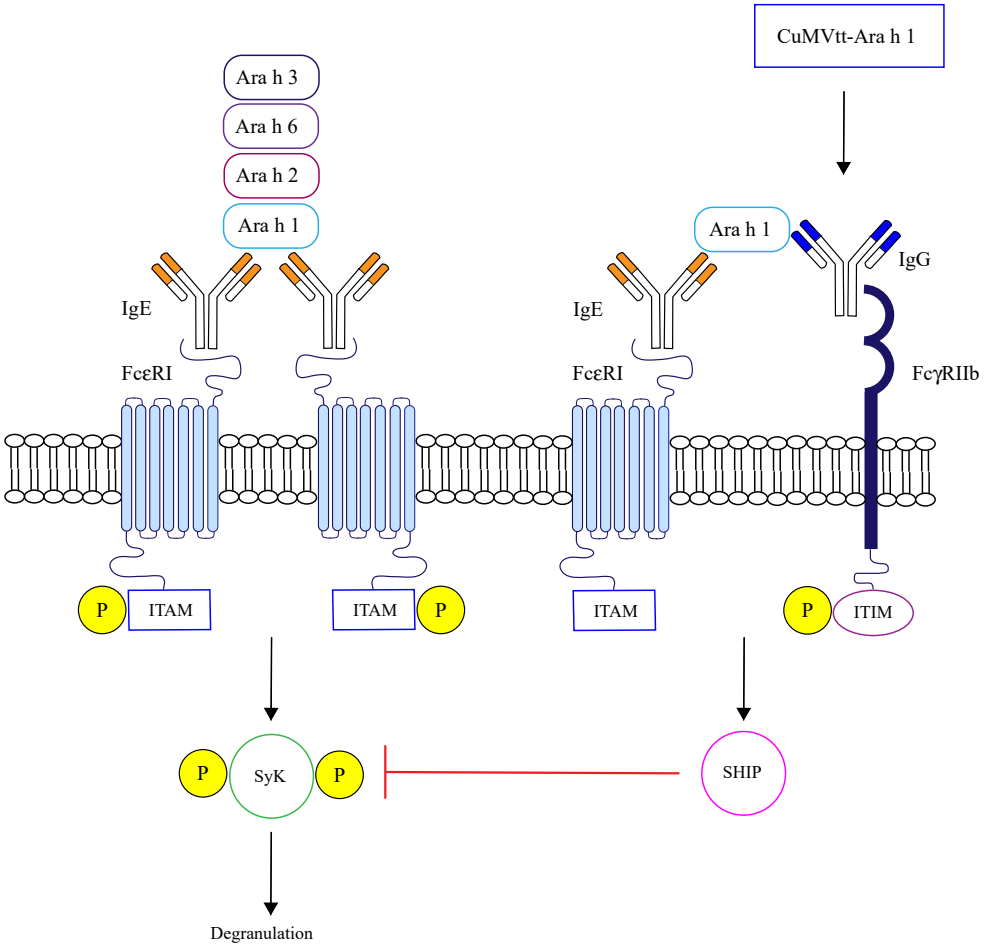

FIG 7. Protection induced by CuMVtt-Ara h 1 vaccine against PA. Proposed mechanism of action by which generated anti–Ara h 1 IgG antibodies stimulate the inhibitory receptor FcγRIIb on mast cells and basophils. FcεRI-meditated degranulation. *ITAM,* Immunoreceptor tyrosine-based activation motif; *ITIM,* immunoreceptor tyrosine-based inhibitory motif; *SHIP,* Src homology domain 2–containing inositol phosphatase; *SyK,* spleen tyrosine kinase. Scheme adapted from Liu et al.[37]

Serum obtained from mice vaccinated with CuMVtt-Ara h 1 inhibits mast cell activation

To confirm *in vitro* the inhibitory effect of vaccination with CuMVtt-Ara h 1 on mast cell activation, bone marrow–derived mast cells were first sensitized with serum obtained from allergic mice. After washing, the challenge was performed with peanut extract preincubated with serum from mice vaccinated with CuMVtt-Ara h 1 or CuMVtt-immunized mice as a control. As shown in Fig 6, *E*, mast cell activation was significantly inhibited by the presence of serum from CuMVtt-Ara h 1 mice, confirming the inhibitory effect of IgG-allergen complexes.

DISCUSSION

This study uses a preclinical setting to test vaccine candidates for PA. The vaccines are based on the immunologically optimized plant VLPs CuMVtt coupled either to peanut extract or to the single major allergens Ara h 1 or Ara h 2. Vaccination against either Ara h 1 or Ara h 2 was sufficient to induce protection against the whole peanut extract consisting of multiple allergens, as assessed in an anaphylaxis model, by skin prick testing and small-bowel eosinophil and mast cell infiltration after gavage.

Efficacy of systemic immunotherapy is thought by some to rely on induction of allergen-specific regulatory T cells[32] or a shift from T_H2 cells toward T_H1 cells,[33] with a consecutive decrease in allergen-specific IgE. Induction of humoral responses with generation of allergen-specific IgG during immunotherapy (increasing the ratio IgG/IgE) is discussed by others as an essential element responsible for induction of allergen tolerance.[34-36] In this model, IgG is supposed to compete with IgE for the allergen, preventing crosslinking of the FcεRI receptor as well as engaging FcγRIIb. A strong "proof of principle" in humans for the protective effect of allergen-specific IgG was obtained through administration of 2 monoclonal Fel d 1–specific IgG antibodies in cat-allergic patients, showing significantly improved symptoms after nasal stimulation tests in a placebo-controlled trial.[20] Therefore, a sufficiently high titer of IgG antibodies with adequate affinity/avidity for the allergen is able to diminish allergic symptoms after exposure. In our study, we found a significant increase in specific IgG responses after CuMVtt-Ara h 1 immunization and demonstrate that

transfer of purified IgG fractions could confer protection against allergic reactions. This supports the role of IgG in the mechanism of protection induced by the vaccine candidates tested here. Moreover, we could show that IgG was not anaphylactogenic itself because transfer of IgG from immunized mice did not induce reactogenicity. This is most likely because high levels of allergens are required for IgG to induce anaphylactic reactions. A limitation of this study is that some experiments were done by way of IV challenge, which is not physiologic for peanut exposure in humans. Nevertheless as previously shown,[22] parenteral injection of allergen may indeed represent a model for systemic exposure, allowing investigations of vaccine-induced protection against systemic symptoms and related mechanisms.

We have shown in this study that vaccination against single allergens results in protection against peanut caused by a complex allergen mixture. Protection was transferrable by IgG antibodies, and the inhibitory receptor FcγRIIb present on mast cells and basophils was critical for reduced allergic symptoms. Fig 7[37] shows a model of the proposed mechanism of action. In allergic patients, peanut allergens engage IgE molecules on mast cells and basophils, causing their activation and the allergic response (Fig 7, left part). In presence of high levels of IgG antibodies specific for a single allergen, IgG-immune complexes will be formed and bind FcγRIIb, causing inhibition of all IgE-mediated signals, including those from IgE molecules cross-linked by other allergens (Fig 7, right part). This explains why IgG antibodies against single allergens are able to block cellular activation by whole allergen extracts. These results are in line with previous studies showing that FcγRIIb was able to inhibit signals generated by activating receptors that were sensitized with non–cross-reacting IgE and were not directly coengaged with FcγRIIb.[38]

Patients' IgE specificities for peanut allergens and even corresponding epitopes can be determined in most cases. This knowledge will enable generating a patient-specific vaccine against the most abundant allergen with high IgE but low IgG responses. Potential cross- reactivity may also be taken into account, because different peanut allergens are known to be cross-reactive.[39,40] Hence, some protection against additional allergens may also be caused by cross-reactivity and regular (cross-reactive) allergen neutralization.

Conclusions

This study delivers a strong preclinical package for a vaccine using a single-peanut-allergen approach displayed on CuMVtt. It combines a desirable safety profile (absence of allergic reactions induced by the vaccine) with an equally attractive efficacy profile because vaccination strongly reduces systemic and local allergic symptoms on challenge with the whole allergen extract. Moreover, using our vaccine approach the fact that immunizing against one single allergen protects against an allergen mixture is striking and could be applied in different relevant allergies, frequently caused by sensitization against more than 1 allergen. In addition, PA is an increasing and severe disease, hence, we highlight the translational potential of this study.

We thank our colleagues from Latvian Biomedical Research and Study Centre, Riga, Latvia—Gunta Resevica, Vilija Zeltina, and Janis Bogans—for elaboration of protocols for peanut allergen purification.

Key messages

- **Peanut allergy is a disease with increasing prevalence, and avoidance of peanut is difficult to achieve.**
- **Oral immunotherapy is effective, but time consuming and potentially dangerous for severely allergic patients.**
- **An active vaccination based on engineered virus-like particles displaying single major peanut allergens generates protective IgG antibodies in a mouse model for peanut allergy.**
- **Protection is FcγRIIb-dependent, showing the critical role of IgG-allergen immune complexes for protection against complex allergen mixtures.**

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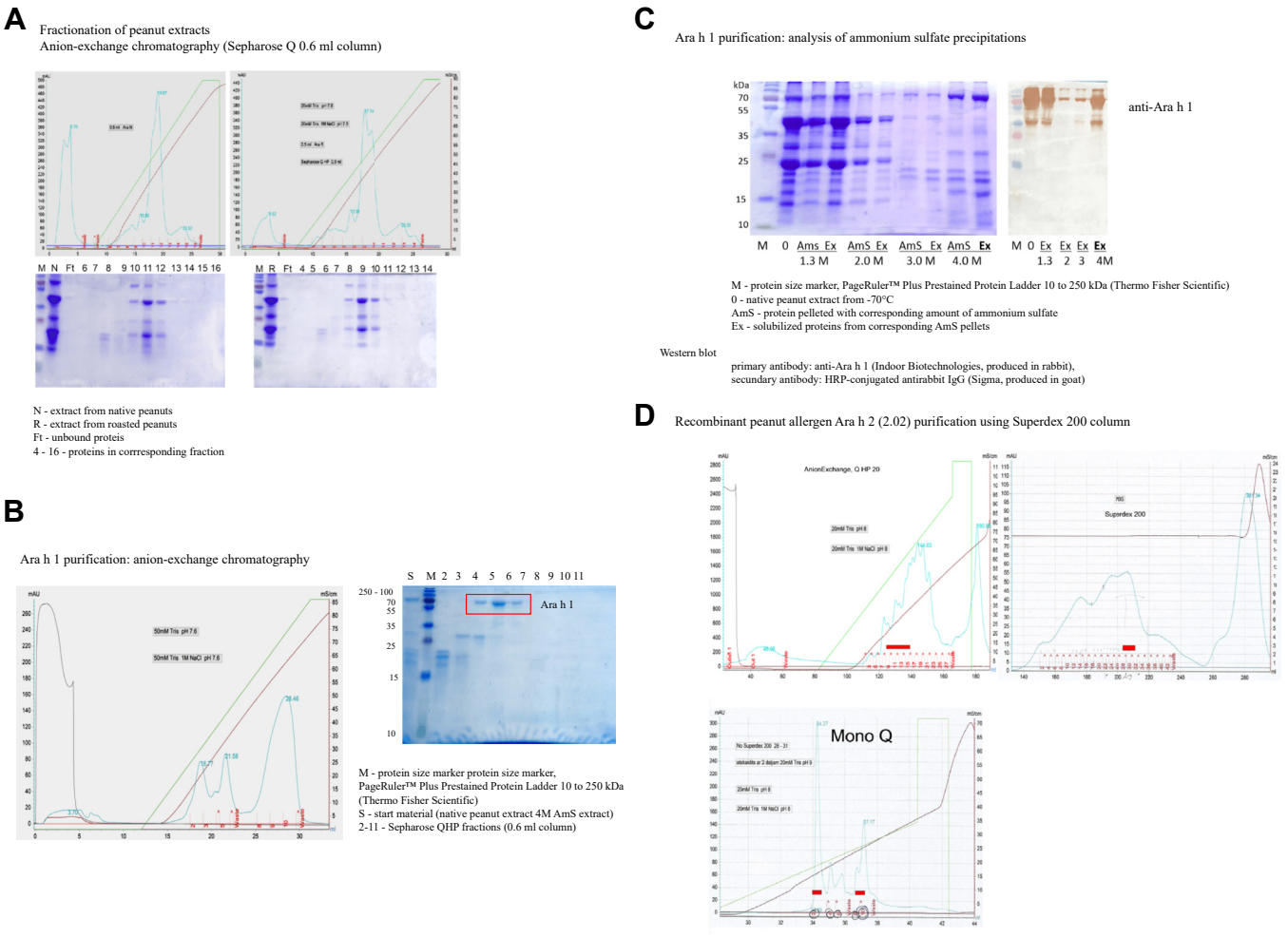

FIG E1. Characterization of Ara h 1. **A,** Enrichment of Ara h 1 by ammonium sulfate precipitation (panel I). Ara h 1 can be enriched by ammonium sulfate precipitation; most part of Ara h 1 is soluble. M, protein size marker (Thermo Scientific; #26619); 0, native peanut extract; AmS, proteins pelleted with corresponding amount (1.3-4 mol/L) of ammonium sulfate; Ex, solubilized proteins from AmS pellets. For Western blots, anti–Ara h 1 (panel II) pAbs from Indoor Biotechnologies were used as primary antibodies and HRP-conjugated antirabbit IgG produced in goat (Sigma) were used as secondary antibodies. **B,** Anion-exchange chromatography of Ara h 1 using Sepharose QHP column (panel I) and SDS-PAGE analysis of Ara h 1 purification (panel II). M, protein size marker (Thermo Scientific; #26619); S, start material (peanut extract after precipitation with 4 mol/L ammonium sulfate); 2-11, Sepharose QHP fractions. **C,** Size-exclusion chromatography of Ara h 1 using Superdex 200 column (panel I). SDS-PAGE analysis of Ara h 1 purification (panel II). M, protein size marker (Thermo Scientific; #26619); 2-11, Superdex 200 fractions. **D,** Dynamic light scattering analysis of purified Ara h 1. Ara h 1 solution (1 mg/mL) was analyzed on a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Malvern, United Kingdom). The results of 3 measurements were analyzed by DTS software (Malvern, version 6.32). The average hydrodynamic diameter ($Z_{(av)}$) was found to be 18.6 nm. *HRP*, Horseradish peroxidase.

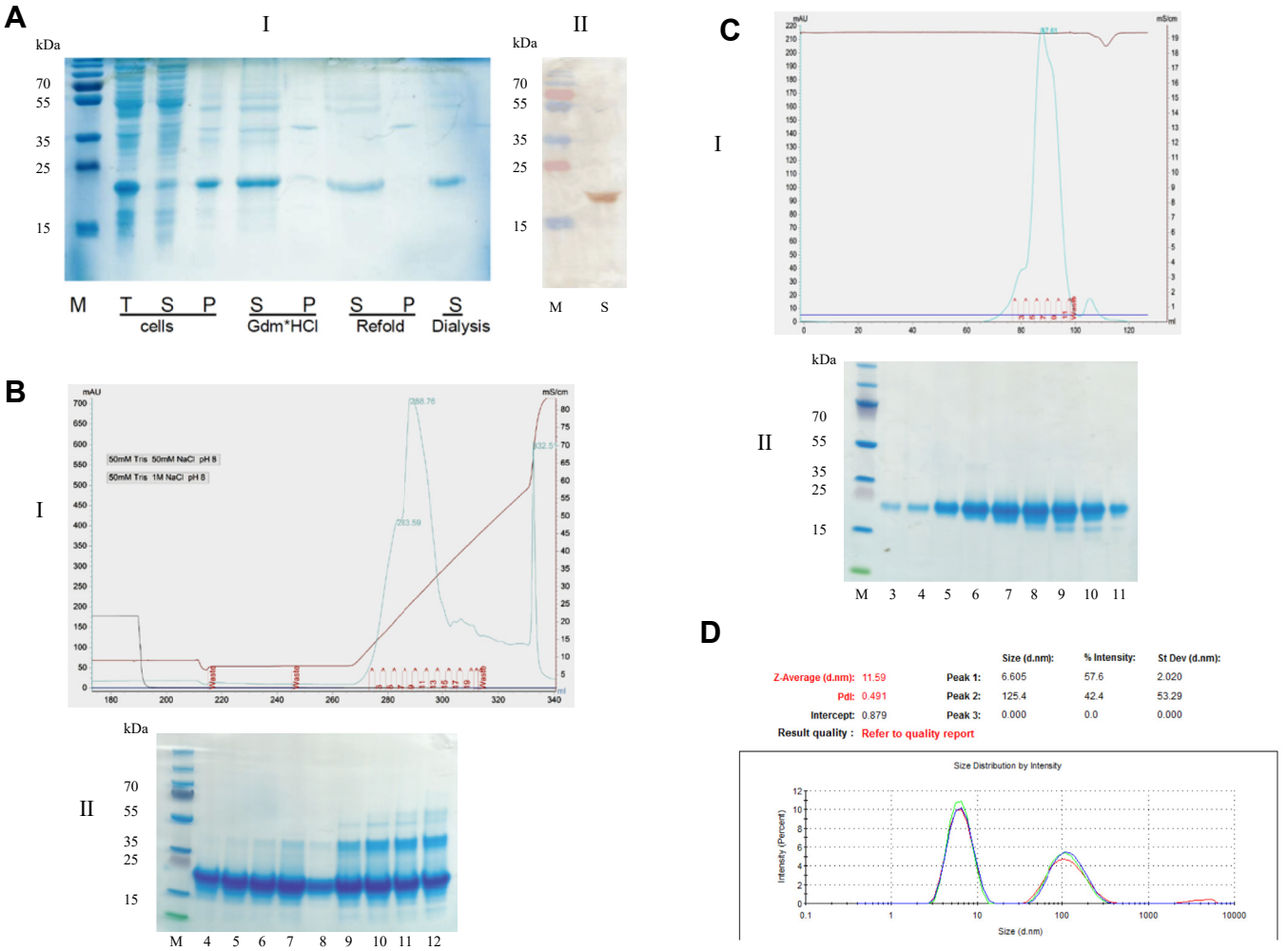

FIG E2. Characterization of Ara h 2. **A**, SDS-PAGE (panel I) and Western blot (panel II) analysis of Arah202 inclusion body solubilization and refolding. M, Protein size marker (Thermo Scientific Page Ruler Plus, #26619); T, total proteins in recombinant *E coli* cells; S, soluble proteins after corresponding treatment; P, insoluble proteins after corresponding treatment. For Western blot, the refolded Arah202 was blotted onto nitrocellulose membrane and treated with Ara h 2 pAbs (Indoor Biotechnologies, produced in rabbits) as primary antibodies; as secondary antibodies, HRP-conjugated antirabbit IgG produced in goat (Sigma) were used. **B,** Anion-exchange chromatography of refolded Arah202 using Sepharose QHP column (panel I). SDS-PAGE analysis of Arah202 purification (panel II). M, protein size marker (Thermo Scientific; #26619); 4-12, Sepharose QHP fractions. **C,** Size-exclusion chromatography of Arah202 using Superdex 200 column (panel I). SDS-PAGE analysis of Arah202 purification (panel II). M, protein size marker (Thermo Scientific; #26619); 3-11, Superdex 200 fractions. **D,** Dynamic light scattering analysis of purified Arah202. Arah202 solution (1 mg/mL) was analyzed on a Zetasizer Nano ZS instrument (Malvern Instruments Ltd). The results of 3 measurements were analyzed by DTS software (Malvern, version 6.32). The average hydrodynamic diameter ($Z_{(av)}$) was found to be 11.6 nm for most part of the protein. Arah202 partially forms stable aggregates greater than 100 nm.

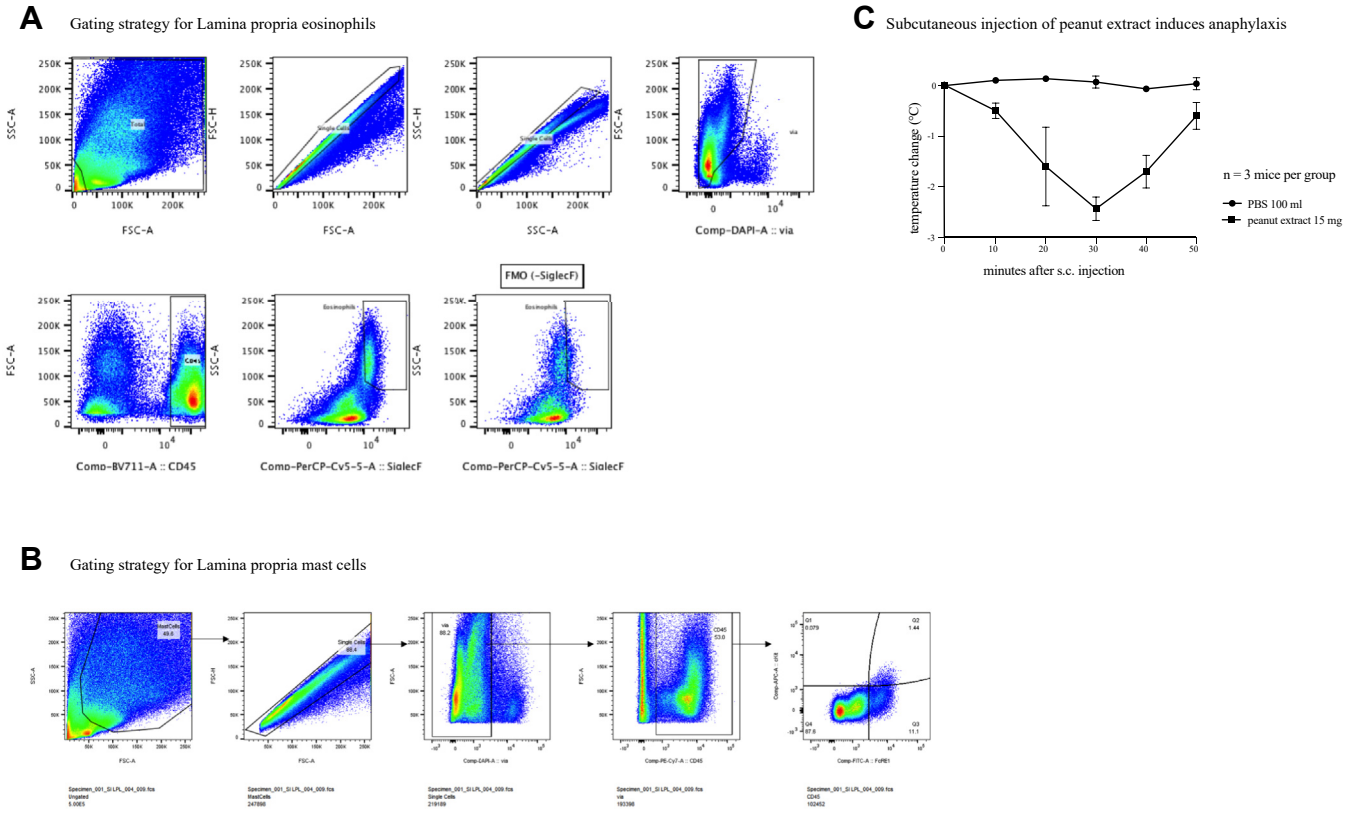

FIG E3. A, Gating strategy for flow cytometric quantification of small intestinal lamina propria eosinophils. Doublets and dead cells were excluded before gating on $CD45^+$ $SiglecF^+$ SSC^{hi} eosinophils. **B,** Gating strategy for flow cytometric quantification of small intestinal lamina propria mast cells. Doublets and dead cells were excluded before gating on $CD45^+$ $FcR\varepsilon1^+$ $cKit^+$ mast cells. **C,** SC allergen injection induces anaphylaxis. To investigate the effect of SC allergen application, mice (n = 3 per group) were injected s.c. with 15-μg dose of free peanut extract (comparable dose of allergen coupled to the CuMVtt used for vaccination). After SC allergen injection, mice develop anaphylactic clinical signs such as immobility and erected hairs and a relevant drop in temperature. *DAPI*, 4′-6-Diamidino-2-phenylindole, dihydrochloride; *FMO*, fluorescence minus one; *FSC-A*, forward scatter; *FSC-H*, forward scatter-height; *SSC-A*, side scatter-area; *SSC-H*, side scatter-height.