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The next generation virus-like particle platform for the treatment of peanut allergy

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Key words: Peanut allergy, novel therapy, vaccine, virus-like particles

ACKNOWLEDGEMENT

This publication was funded by Allergy Therapeutics PLC, Saiba AG, the Swiss National Science Foundation (SNF grants 31003_185114 and IZRPZO_194968). Open access funding provided by Inselspital, University Hospital Bern.

CONFLICT OF INTERESTS

M. D. Heath, T. L. Carreno Velazquez, M. F. Kramer, C. J. W. Scott and M. A. Skinner are employed by Allergy Therapeutics (ATL) PLC. ATL develop and manufacture allergen immunotherapy products and diagnostics. ATL have a license agreement with Saiba AG to develop and commercialise the CuMV$_{TT}$ VLP platform for allergy. M. F. Bachmann is a board member of Saiba AG and holds the patent of CuMV$_{TT}$. J. M. Sobczak and M. O. Mohsen received payments by Saiba AG to work on the development of vaccines against Dengue Fever in the framework of a Eurostars grant and SARS-CoV-2. M. F. Bachmann, M. O. Mohsen and T. M. Kündig are shareholder of Saiba AG.

AUTHOR CONTRIBUTIONS

FS, JMS, AZ, IB, MDH, MFK, TMK and MFB: design of experiments and methodology. JMS, PSK, IB, AZ, MDH, TLCV, MFK, MV and MFB: acquisition of data, analysis and interpretation.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.15704

JMS, MDH, TLCV, MFK, CJWS, MV, and MFB: Writing, revision and editing of manuscript. IB, GR, AZ, MDH, TLCV, MFK, MAS, JMS, MOM and MFB: Technical, material and tool support. MOM, MV, MDH, TLCV, MFK, AZ and MFB: Study supervision. All authors read and approved the final manuscript.

Abstract

Background: Allergy to peanut is one of the leading causes of anaphylactic reactions among food allergic patients. Immunization against peanut allergy with a safe and protective vaccine holds a promise to induce durable protection against anaphylaxis caused by exposure to peanut. A novel vaccine candidate (VLP Peanut), based on virus-like particles (VLPs), is described here for the treatment of peanut allergy.

Methods and Results: VLP Peanut consist of two proteins: a capsid subunit derived from *Cucumber mosaic virus* engineered with a universal T cell epitope ($CuMV_{TT}$) and a $CuMV_{TT}$ subunit fused with peanut allergen Ara h 2 ($CuMV_{TT}$-Ara h 2), forming mosaic VLPs. Immunizations with VLP Peanut in both naïve and peanut-sensitised mice resulted in a significant anti-Ara h 2 IgG response. Local and systemic protection induced by VLP Peanut were established in mouse models for peanut allergy following prophylactic, therapeutic and passive immunizations. Inhibition of FcγRIIb function resulted in a loss of protection, confirming the crucial role of the receptor in conferring cross protection against peanut allergens other than Ara h 2.

Conclusion: VLP Peanut can be delivered to peanut-sensitized mice without triggering allergic reactions, whilst remaining highly immunogenic and offering protection against all peanut allergens. In addition, vaccination ablates allergic symptoms upon allergen challenge. Moreover, the prophylactic immunization setting conferred the protection against subsequent peanut-induced anaphylaxis, showing the potential for preventive vaccination. This highlights the effectiveness of VLP Peanut as a prospective break-through immunotherapy vaccine candidate towards peanut allergy. VLP Peanut has now entered clinical development with the study PROTECT.

1. Introduction

Allergy is defined as a specific hyperimmune response that occurs following exposure to a given allergen, eventually causing an adverse health effect[1]. A particularly dangerous manifestation of allergy is systemic anaphylaxis. Anaphylaxis is a life-threatening, rapid, systemic hypersensitivity reaction, classically mediated by interaction between allergen and allergen-specific Immunoglobulin (Ig) E, leading to degranulation of mast cells and basophils[2]. As shown in previous studies, food allergies are frequently associated with anaphylaxis[3]. Indeed, in the case of peanut, exposure to very low traces of the peanut allergen can result in severe allergic reactions such as anaphylaxis and death[4]. The prevalence of peanut allergy in Western countries ranges between 1.4 to 3% in children and is steadily increasing over the years[5]. While some food allergies such as egg and milk can be outgrown, peanut allergy is usually a lifelong disease. Consequently, this allergy affects the quality of life for patients and their families with a continuous fear of accidental ingestion of peanut[6].

Of those with peanut allergy in the US, almost 60% have experienced a severe reaction and nearly three-quarters (73.0%) of those with peanut allergy also have an epinephrine prescription for treatment of an anaphylactic reaction. Moreover, 50% have had at least one lifetime emergency department visit, with 23% having had an emergency department visit in the previous year[7]. Thus, an effective treatment for peanut allergy is a high unmet medical need. Peanuts (*Arachis hypogaea*) belong to the *Leguminosae* family and contain 17 different allergens and their isoforms (WHO/IUIS database: allergen.org)[8]. The sensitization patterns to peanut allergens are geographically heterogenous, with 2S albumin Ara h 2 being a dominant allergen both in the USA[9] and Europe[10]. Other major peanut allergens include other soluble storage proteins Ara h 1 and Ara h 3[11], as well as Ara h 6 (homologous to Ara h 2)[12] and insoluble structural proteins (oleosins) (Ara h 10, Ara h 11, Ara h 14 and Ara h 15)[13]. Although immunoglobulin (Ig) E specificities vary among peanut allergic patients, 97% of the individuals with peanut allergy are sensitized to at least one of the storage proteins[14]. Clinically, peanut allergy associated with sensitization to Ara h 2 and Ara h 6 usually develops into severe forms[15].

Ara h 2 and Ara h 6 are the most frequently and strongly recognized antigens in peanut-allergic children, as determined by both immunoblot[16] and skin prick test (SPT)[17], as well as the most frequently recognized and potent peanut allergens in a group of well-characterized peanut-allergic patients[18]. As Ara h 2 could be the most important mediator of peanut allergic reactions, it may be a key allergen for use in allergen-specific immunotherapy (AIT)[19].

Although immunotherapy can induce changes in levels of IgE and IgG4 to different peanut allergens, a recent study showed that changes in antibody titer in response to peanut AIT were predominantly to Ara h 2 and Ara h 6, with significant increases in Ara h 2 and Ara h 6 IgG4/IgE[20,21]. This again highlights the importance of these allergens in peanut allergy and AIT.

There are several methods available to treat IgE-mediated allergies. The most accessible and commonly used therapies are symptomatic in nature and block allergic reactions at the effector stage by inhibiting the action of histamine and/or leukotrienes. While these are effective, they are not without chronic adverse effects and do not stop the progression of the disease or modify its state to address the underlying cause[22]. Other conventional approaches aim to facilitate a state of desensitization, the principal goal of disease modifying immunotherapies, by exposing the participant to increasing doses of allergen and, therefore, raising the threshold of reaction[23]. In certain patients a sustained unresponsiveness to food allergen may be achieved following immunotherapy; however, the long-term efficacy of protection is highly questionable[24]. Despite some clinical successes, conventional AIT based on allergen desensitization protocols (oral, sublingual, subcutaneous) lacks validated and generally accepted candidate biomarkers that are predictive or indicative of the clinical response to AIT.

It is well established that classical AIT induces allergen-neutralizing IgG antibodies[25,26,27] and that these antibodies can block IgE-mediated antigen presentation in a competitive high affinity and epitope-specific matter. In addition, it has been shown in murine models of cat or peanut allergies that the transfer of allergen-specific IgG induced by allergy vaccination resulted in protection against local and systemic allergen challenge[28,29] in a non-competitive low affinity pathway via inhibitory FcγRIIb activation. Thus, allergen-specific IgG antibodies may block mast cell and/or basophil activation via distinct pathways through direct allergen neutralization[30], co-internalization of IgE by mast cells[31] and engagement of the inhibitory Fc gamma receptor IIb (FcγRIIb)[28,32]. Binding of allergen-IgG immune complexes to FcγRIIb, mainly expressed by mast cells and basophils[33], induces phosphorylation of the SHIP (SH2-containing-phosphatidylinositol-5'-phosphatase) phosphatase leading to dephosphorylation and consequent deactivation of the FcεRI pathway[31,34] which results in inhibition of cellular activation. Notably, this inhibition of allergy effector cells via FcγRIIb activation requires only one allergen to protect against all other co-exposed allergens – even beyond structural homology. Further, it has previously been shown that blocking FcγRIIb with an anti-FcγRIIb monoclonal antibody (mAb) suppresses protection against anaphylaxis in peanut sensitized mice actively immunized with a VLP based peanut vaccine candidates[35] as well as passively immunized with IgG induced by these vaccines[29].

A re-emerging concept to treat allergy is centered on the protective umbrella of allergen specific IgG antibodies, which may be considered key biologic mediators in vaccination but

also AIT that could be optimally induced by combining allergens with nanoparticle technologies[36]. Integrating allergens within virus-like particles (VLPs) harnesses the properties of a viral structure to stimulate the production of allergen-specific IgG antibodies and provides protection against peanut allergy (PA) without the risk of triggering the disease (i.e., eliminating its allergic potential). While VLP-based vaccines are highly immunogenic, nonclinical data indicate that they have strongly reduced potential to activate human mast cells due to impaired ability to bind to surface-bound IgE[37]. This is due to the size, geometry and diffusion kinetics of VLPs within tissues[38], which ultimately disfavours FcεRI-mediated signals[22]. Importantly, VLPs can be used as a vector to both display and carry antigens on the VLP surface, thus exploiting evolutionary traits for optimal anti-viral immune recognition[39,40]. This is particularly important if the goal of therapeutic vaccination is to reprogram towards a Type 1 (non-IgE) mediated response to the allergen.

Vaccine candidates for peanut allergy treatment based on a virus-like particle (VLP) platform were previously described by our group[35]. Vaccines generated by displaying of peanut allergens on the CuMV$_{TT}$ VLP via chemical coupling proved to be highly immunogenic and were effectively protective against local and systemic anaphylaxis in a peanut allergy mouse model[35]. In the current study, a next-generation vaccine candidate (VLP Peanut) for the treatment of peanut allergy is described. VLP Peanut consists of two proteins: a capsid subunit protein (CuMV$_{TT}$) and a capsid subunit protein genetically fused with major peanut allergen Ara h 2 (CuMV$_{TT}$-Ara h 2), spontaneously assembling together to form mosaic VLPs (CuMV$_{TT}$/CuMV$_{TT}$-Ara h 2). The use of a genetic fusion makes the vaccine scalable and relatively inexpensive to manufacture, whilst maintaining all its main features, such as immunogenicity and long-term stability[41,42]. More importantly, the use of a fusion VLP is critical for patient safety, since this strategy minimizes the risk of presence of trace amounts of “free” Ara h 2 allergen in the vaccine sample.

2. Materials and methods

Detailed description of materials and methods is provided in the Appendix S1.

Production and purification of $CuMV_{TT}$ VLPs

Cucumber mosaic virus (CMV) coat protein (CP) gene was sub-cloned into the pET28a(+) expression plasmid. The tetanus toxoid (TT) epitope sequence was introduced into the CMV CP gene by 2-step PCR mutagenesis. For expression, E. coli C2566 cells were transformed with the pETCuMV$_{TT}$ plasmid containing the CuMV$_{TT}$ CP gene (CuMV$_{TT}$ – CMV derived VLPs). Biomass was collected and the cells were disrupted by sonication in a lysis buffer. The CuMV$_{TT}$ VLPs precipitated overnight at 4°C in a buffer containing 3 M ammonium sulfate. Subsequently, samples were centrifuged and pellets dissolved in a sodium borate buffer. The solution containing CuMV$_{TT}$ particles was ultracentrifuged in a sucrose gradient. Gradient fractions were analyzed by SDS PAGE. Appropriate fractions containing CuMV$_{TT}$ VLPs were ultracentrifuged twice through 30% sucrose "cushion" for LPS removal. Pellets obtained were diluted in VLP storage buffer and stored at 4°C[35,43]. The quality control of the CuMV$_{TT}$ batch was performed with SDS-PAGE analysis (Fig. S1A, lane 2), agarose gel and TEM. The quantification of CuMV$_{TT}$ VLPs protein content was performed with a use of Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, cat. 23227).

VLP Peanut ($CuMV_{TT}$/$CuMV_{TT}$-Ara h 2) vaccine production and purification

CMV-Ntt830 gene was inserted within the polylinker of pETDuet-1 and amplified by PCR. After amplification, the corresponding PCR product was cloned into the pTZ57R/T vector. *E. coli* XL1-Blue cells were used as a host for cloning and plasmid amplification. A pTZ-plasmid clone containing a CMV-Ntt830 gene without sequence errors was cut with HindIII, treated with Klenow enzyme and NdeI restrictase. The fragment was then sub-cloned into the pETDuet-1, resulting in the helper vector pETDu-CMV-Ntt830. The CMVB2xArah202 insert was excised from pACYCDu-CMVB2xArah202 and subcloned to the helper vector. The plasmid clone with the correct fragment pattern was identified and designated as pETDu-CMVB2xArah202-CMVNtt830.

E. coli C2566 competent cells were transformed with the plasmid pETDu-CMVB2xArah202-CMVNtt830 and grown in 2TY medium containing ampicillin on a shaker (10 rpm, 30°C) to an OD600 value between 0.8–1.0. Cultures were induced with 0.2 mM IPTG and the medium was supplemented with 5 mM $MgCl_2$ for enhancement of biomass growth and protein expression[44-46]. Incubation continued on a shaker (10 rpm, 20°C, 18 h).

To purify mosaic CMVxArah202/CMV-Ntt830 VLPs 6 g biomass was suspended in 20 mL of lysis buffer and sonicated. The insoluble material was removed by centrifugation. The VLP sample (5 mL) was overlaid onto a sucrose gradient (20-60%) and ultracentrifuged. The

content of each gradient was analyzed by SDS-PAGE. Analysis suggested the presence of mosaic VLPs in a fraction containing 40% sucrose. That fraction was diluted 1:1 in sodium borate buffer and VLPs were collected by another ultracentrifugation. Pellets were solubilized in 3 mL of a sodium borate buffer. For the LPS removal, the solution containing VLPs was overlaid onto a 20% sucrose "cushion". VLPs were subsequently collected by ultracentrifugation followed by solubilization in a sodium borate buffer. Ultracentrifugation through a sucrose "cushion" was repeated and the VLPs were solubilized in a VLP storage buffer. The Western blot analysis has been performed to demonstrate the absence of free Ara h 2 in the VLP Peanut sample (Fig. S1B). The quantification of VLP Peanut protein composition was performed with a use of Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, cat. 23227). The proper folding of antigen, its density and exposure to the outer site of the VLPs was tested with sandwich ELISA assay (Fig. S1C).

Animals

Mouse experiments were conducted using female BALB/cOlaHsd mice (8-12 weeks old) applying protocols approved by the Swiss Cantonal Veterinary Office (license no. BE 70/18).

Immunization of naïve and sensitized mice

To test the immunogenicity of the vaccine, naïve or sensitized mice were immunized twice at an interval of 14 days. All immunizations were performed subcutaneously (s.c.). Preliminary immunizations for the dose selection were performed with 3 µg, 10 µg, 30 µg, 100 µg of VLP Peanut, diluted in PBS to a final volume of 200 µL. These doses were chosen based on previously published works from our group utilizing VLP-based vaccines[35,41,42,47,48]. All subsequent immunizations were performed subcutaneously (s.c.) with 30 µg of VLP Peanut or $CuMV_{TT}$, diluted in PBS to a final volume of 200 µL.

Mice sensitization to peanut

Naïve or immunized mice were sensitized to peanut with two intraperitoneal (i.p.) injections containing 5 µg of roasted peanut extract (Ara R) formulated in Alum. Injections were performed at an interval of 7 days.

Systemic challenge

To induce systemic anaphylaxis and test vaccine efficacy, mice were pre-warmed in a Small Warm Air System chamber (Vet-Tech, Congleton, UK; cat. HE011AR) for 10 min at 38.5°C and, subsequently, injected intravenously (i.v.) with 20 µg of Ara R diluted in PBS. Systemic anaphylaxis was assessed by measuring mice body temperature[49-51] prior to challenge as well as up to 50 min after i.v. injections, at intervals of 10 min.

Skin prick test

A skin prick test (SPT) was used to assess local effects of the vaccine. Sensitized and immunized mice were given i.v. injections of Evans blue solution[52]. Thirty minutes later ear skin pricks were performed using a needle and a drop of peanut extract solution was placed onto the skin of the outer ear of anesthetized mice.

Passive IgG transfer

Mouse IgGs for passive vaccination were induced by two immunizations of 20 naïve mice with 50 µg of VLP Peanut or CuMV$_{TT}$ at an interval of 14 days. On day 28, mice were terminally bled and pooled sera for both groups were collected. Total serum IgGs were purified. In parallel, another 20 mice were peanut-sensitized. Two weeks after sensitization, mice were split into 5 groups and received a passive transfer of purified IgGs from sera of mice immunized with VLP Peanut via i.v. injection (25 µg, 50 µg, 75 µg & 100 µg/mice). Control mice received 150 µg of purified IgGs from sera of mice immunized with CuMV$_{TT}$. Systemic challenge with Ara R was performed 24 h after passive IgG transfer.

FcγRIIb inhibition

To investigate the role of the inhibitory receptor FcγRIIb in the protection against anaphylaxis, 2 groups of peanut-sensitized mice received a passive transfer of 150 µg of IgGs. IgGs used for passive vaccination were isolated from mice immunized with 50 µg of VLP Peanut. In addition, the control group received 150 µg of an anti-FcγRIIb mAb (AT 128, provided by M. Cragg, Antibody and Vaccine Group, Southampton, UK)[29,35]. Systemic challenge with Ara R was performed 24 h after passive IgG transfer.

ELISA for determining anti-Ara h 2 IgG

96-well plates were coated with 2 µg/mL of Ara h 2 protein. Mouse sera were added to the plates with a starting dilution of 1:10 in PBS-Casein 0.15%. Serial dilutions of pre-diluted sera were performed with a dilution ratio of 1:3. Subsequently, a goat anti-mouse IgG, HRP (1:5000) was used as a secondary antibody. After ELISA development, plates were read at an OD of 450 nm (OD_{450}). Half-maximal antibody titers were defined as the reciprocal of the dilution leading to half of the OD measured at saturation.

For determination of anti-Ara h 2 specific IgG subclasses, the following detection antibodies were used: goat anti-mouse IgG1, HRP and goat anti-mouse IgG2a, HRP (1:1000), goat anti-mouse IgG2b, HRP (1:4000), rat anti mouse IgG3 (1:2000), Biotin Rat Anti-Mouse IgG3 (1:2000). IgG subclasses were measured from sera pre-diluted 1:20.

ELISA for determining anti-Ara h 2 IgE

ELISA plates were prepared as described in previous section. Mouse sera (10 µL per sample) were diluted 1:10 in PBS-Casein 0.15% up to a final volume of 100 µL. Each diluted serum sample was added to pre-washed magnetic beads (Dynabeads™) coupled with G protein, originating initially from the 250 µL bead suspension. Samples were incubated in Eppendorf tubes on rotator for 1 h at RT. After the incubation period, pre-diluted sera samples were isolated from the beads using a DynaMag-2 magnet and applied directly onto the ELISA plates, before diluting down the plate with a dilution factor of 1:2. Plates were incubated at 4°C with agitation, overnight. A goat anti-Mouse IgE Antibody, HRP (1:1000) was used as a secondary antibody on the following day. Endpoint titers were defined as the reciprocal of the serum dilution leading to the cut-off value of OD_{450} = 0.13; *Cutoff* = (MEAN + 3 x SD) of three negative control (naïve serum) measurements[53,54].

Competitive ELISA

ELISA plates were prepared as described in previous sections. Mouse sera were added to the top wells of the plates with a starting dilution of 1:5 in PBS-Casein 0.15% containing constant concentration of 250 ng/mL of anti-Ara h 2 IgE (P12P3D08)[55], expressed and purified as described in Beerli et al., 2008[56]. Next, serial dilutions of pre-diluted sera was performed down the plates with dilution ratio of 1:2. Plates were incubated for 1.5 hour shaking at RT. A goat anti-Mouse IgE Antibody, HRP (Bethyl Laboratories, Montgomery, TX, USA; cat. A90-115P) diluted 1:1000 in PBS-Casein 0.15% was used as a secondary antibody.

3. Results

Technical characterization of VLP Peanut

VLP Peanut (CuMV$_{TT}$/CuMV$_{TT}$-Ara h 2) consist of two proteins; a capsid subunit protein (CuMV$_{TT}$) and capsids genetically fused with Ara h 2 (CuMV$_{TT}$-Ara h 2). Subunits are expressed within *E. coli* and spontaneously assemble to form mosaic VLPs (CuMV$_{TT}$/CuMV$_{TT}$-Ara h2) with a diameter of about ~36 nm (see outline in Fig. 1A). The successful expression of CuMV$_{TT}$ VLPs in a bacterial culture preserves native T = 3 icosahedral geometry[40] of *Cucumber mosaic virus* (CMV) consisting of 180 coat protein subunits[40,57,58], efficiently draining to secondary lymphoid organs[38]. The morphology of VLP Peanut was confirmed by transmission electron microscopy (TEM) (Fig. 1B). As shown by SDS-PAGE, the capsid subunit protein (CuMV$_{TT}$) has molecular weight of ~28 kDa, whereas the capsid subunit genetically fused with Ara h 2 (CuMV$_{TT}$-Ara h 2) has molecular weight of ~45 kDa (Fig. 1C). The presence of prokaryotic nucleic acid (RNA) inside the VLPs has been shown using agarose gel electrophoresis (Fig. 1D). DLS analysis shows the presence of a homogeneous peak with an average hydrodynamic diameter (D_h) of about ~44 nm for VLP Peanut (Fig. 1E).

Immunization with Ara h 2-fused, mosaic VLP Peanut induces anti-Ara h 2 IgG and anti-Ara R responses in naïve mice

The humoral response upon immunization with VLP Peanut was investigated in naïve BALB/c female mice according to the scheme shown in Fig. 2A. Animals were vaccinated via the subcutaneous (s.c.) route on day 0 and boosted on day 14. First, a preliminary IgG dose response experiment, with vaccine doses of 3 µg, 10 µg, 30 µg and 100 µg, was performed. Sera of immunized mice were collected on day 28 and the levels of antigen specific anti-Ara h 2 total IgG were examined by ELISA. The result revealed no significant difference in anti-Ara h 2 IgG specific responses between two highest doses used: 30 µg and 100 µg, however these were significantly higher than responses measured in mice immunized with 3 µg and 10 µg of a vaccine (Fig. S2A). Based on this result, we chose a dose of 30 µg VLP Peanut formulated in 200 µl PBS buffer was selected. The immunization with the selected 30 µg dose was repeated and sera from experimental mice was collected every week until day 28, starting from the pre-immunization time point on day 0. The elicited anti-Ara h 2 IgG response was detected as early as day 7 for mice vaccinated with VLP Peanut followed by a significant (>10-fold) increase on day 14. Moreover, the booster dose augmented the anti-Ara h 2 IgG response by approximately another 10-fold on day 28 (Fig. 2B). Similar levels of IgG specific to roasted peanut extract (Ara R) were detected, reaching significant, nearly 10-fold increase on day 14, followed by another 10-fold increase on day 28 (Fig. S2B). Finally, the anti-Ara 2 IgG levels

induced by 30 µg of VLP Peanut were compared to those induced by 30 µg of $CuMV_{TT}$ chemically coupled to Ara h 2, where no significant differences could be detected (Fig. S2C).

IgG responses are dominated by the IgG1 and IgG2a/IgG2b subclasses and enhanced by priming with TT prior to immunization

Previous studies have shown some of the IgG subclasses, or their constant parts, are important constituents in the process of desensitization for allergic patients[19,59,60] and allergic mouse models[61-63]. Therefore, it was prudent to assess the ability of VLP Peanut to induce all Ara h 2-specific IgG subclasses: IgG1, IgG2a, IgG2b and IgG3. Serum titers for mice vaccinated with VLP Peanut for all four anti-Ara h 2 IgG subclasses were significantly higher when compared to mice vaccinated with control VLPs ($CuMV_{TT}$) 2 weeks post-vaccination. Further analysis of $Log_{10}OD_{50}$ data showed a dominant response of anti-Ara h 2 IgG1 subclass two weeks after the booster dose, followed by slightly lower anti-Ara h 2 IgG2a and IgG2b responses (Fig. 2C).

To assess, whether the incorporation of tetanus toxoid (TT) epitope in the structure of VLP Peanut is able to enhance the specific anti-Ara h 2 IgG response, mice were primed with 10 µg of TT formulated in 100 µl of Alum as shown in a scheme (Fig. S2D). Indeed, mice primed with TT had higher serum levels of anti-Ara h 2 IgG at each time point measured throughout the experiment in comparison to control mice primed with PBS, reaching the statistically significant difference two weeks after the boost (Fig. S2E).

IgG antibodies induced by immunization with VLP Peanut successfully compete for an Ara h 2 epitope with anti-Ara h 2 IgE mAb

The capacity of VLP Peanut-induced anti-Ara h 2 IgG to inhibit the binding of anti-Ara h 2 IgE to Ara h 2 was tested using competitive ELISA assay. The ELISA for detection of anti-Ara h 2 IgE bound to Ara h 2 was performed with the constant concentration of 250 ng/mL of monoclonal anti-Ara h 2 IgE (P12P3D08)[55] present in the dilution buffer and with serial dilutions of serum collected on days 14 and 28 from mice immunized with VLP Peanut or $CuMV_{TT}$ as control. The sequence of anti-Ara h 2 IgE (P12P3D08) was obtained from Croote et al. 2018[55] and an expression plasmid was generated by gene-synthesis. The result clearly demonstrates the ability of serum from mice immunized with VLP Peanut to directly inhibit the binding of IgE to Ara h 2 (Fig. 2D).

Challenge with VLP Peanut do not induce local and systemic adverse effects in peanut-sensitized mice

Local and systemic reactogenicity of the VLP Peanut vaccine was evaluated in peanut-sensitized mice using skin prick test and i.v. challenge, respectively, performed with VLP Peanut, $CuMV_{TT}$ (negative control) and peanut extract (positive control) according to the

regimen shown in Fig. S3A. Each challenge was performed with a ratio of 3:1 of the vaccine concentration to the concentration of positive control peanut extract in order to consider the equivalent ratio of administered peanut allergens[35].

To assess the impact of VLP Peanut on local allergic reaction, peanut-sensitized BALB/c mice were pre-treated by i.v. injection with 200 µL of Evans Blue dye solution to allow visualization of skin prick tests. Following the administration of Evans Blue dye (30 min), skin prick tests were performed with 23G needles. Next, a drop of either VLP Peanut (1.5 mg/mL) or $CuMV_{TT}$ (1.5 mg/mL) or peanut extract (0.5 mg/mL) was placed onto the outer ear skin of each anesthetized mouse. Animals pricked with VLP Peanut or $CuMV_{TT}$ showed significantly smaller vascular leakage of the dye in comparison to those pricked with peanut extract (Fig. S3B).

To measure the impact of VLP Peanut on systemic allergic reaction, peanut-sensitized BALB/c mice were challenged via i.v. route with either 30 µg of VLP Peanut or 30 µg of $CuMV_{TT}$ or 10 µg of peanut extract. To assess whether i.v. challenge with VLP Peanut did not induce inexpedient anaphylactic reaction, a reference body temperature was recorded just prior to i.v. injection challenge (T=0 min) followed by its measurements at 10-minute intervals for 50 minutes. In line with the result obtained with the skin prick test, animals challenged i.v. with peanut extract developed significantly higher challenge-dependent hypothermia than those vaccinated with VLP Peanut or $CuMV_{TT}$ (Fig. S3C).

Vaccination with VLP Peanut confers systemic protection

Vaccine efficacy and vaccine dose-dependency were assessed by its therapeutic ability to protect peanut sensitized mice against anaphylaxis during systemic challenge. In line with a mouse model for peanut allergy previously established by our group[35], naïve BALB/c mice were first sensitized twice to peanut and then immunized either once (on day 35) or twice (on days 21 and 35) with 30 µg of VLP Peanut via s.c. route. In addition, the control group was primed and boosted with 30 µg of unmodified $CuMV_{TT}$. Two weeks after the last vaccine dose had been administered, mice were challenged via i.v. injection with 20 µg of Ara R. Furthermore, to examine the longevity of protection, a second i.v. injection challenge was performed 30 days after the first challenge (Fig. 3A).

When quantifying the anti-Ara h 2 IgG response induced by VLP Peanut during this therapeutic regimen, a significantly higher serum level was observed following both prime and booster doses of the vaccine in contrast to the control group (Fig. 3B). Data confirms that the vaccine maintained a high level of immunogenicity in mice previously sensitized to peanut. A significant difference between anti-Ara h 2 IgG serum levels of groups immunized with VLP Peanut and $CuMV_{TT}$ was still observed after the first i.v. challenge (Fig. 3C).

To assess whether vaccination with VLP Peanut protected mice against anaphylaxis, a reference body temperature was recorded just prior to i.v. injection challenge (T=0 min) followed by its measurements at 10-minute intervals for 50 minutes. As shown in Fig. 3D, systemic, challenge-dependent hypothermia was significantly higher in the control group than in the mice vaccinated with VLP Peanut. Moreover, the result indicated a vaccine dose-dependent manner of the temperature drop, with higher protection against challenge-dependent hypothermia in mice that received two doses of the vaccine than in mice which received only a single dose. Likewise, during the second i.v. injection challenge performed 30 days after the first challenge, the same protection pattern was maintained, with only slightly smaller significance levels (Fig. 3E).

Vaccination with VLP Peanut prevents an increase of anti-Ara h 2 IgE serum titers following systemic challenge

The modulation and kinetics of specific IgE in response to different immunotherapies is not yet well understood. For instance, in a clinical study published by Aasbjerg and co-workers[64], some evidence indicates that sub-lingual immunotherapy (SLIT) used for the treatment of allergic rhinitis patients may initially induce a significant burst of specific IgE levels followed by their constant decline, also during the following pollen season[64]. On the other hand, the allergen-specific IgE response to sub-cutaneous immunotherapy (SCIT) of allergic rhinitis, presented in the same study, was significantly lower than that induced with SLIT as well as significantly lower when compared to controls after the pollen season. These lower allergen-specific IgE levels in SCIT treated patients, were maintained for a number of months, until the end of the study[64]. To check whether this phenomenon replicates in our study in mice, we measured serum Ara h 2-specific IgE levels at specific time-points during therapeutic regimen: after sensitization, after immunization as well as two weeks after systemic challenge. In agreement with the outcomes described by Aasbjerg and co-workers in the clinical study abovementioned[64], our results revealed no significant changes in anti-Ara h 2 IgE levels two weeks after i.v. challenge for groups of mice immunized with VLP Peanut, irrespective of the number of vaccine doses applied. In contrast, anti-Ara h 2 IgE titers significantly increased following i.v. injection challenge in mice mock-vaccinated with control VLPs ($CuMV_{TT}$). Moreover, the measured Ara h 2-specific IgE endpoint titers before and after immunization were not significantly different (Fig. 3F).

VLP Peanut protects against adverse local response

Having demonstrated that VLP Peanut is able to protect against systemic anaphylaxis, the impact of therapeutic vaccination on local allergic reactions was investigated using skin prick test. Peanut sensitized BALB/c mice were immunized s.c. either with 30 µg of VLP Peanut or

with 30 µg of $CuMV_{TT}$ (control) on days 21 and 35, according to the regimen shown in Fig. 4A. On day 50, prior to performing the local challenge, mice were pre-treated by i.v. injection with 200 µL of Evans Blue dye solution to allow visualization of skin prick tests. Following the administration of Evans Blue dye (30 min), skin prick tests were performed with 23G needles and a drop of Ara R was placed onto the outer ear skin of each anesthetized mouse. In consensus with systemic challenge, animals vaccinated with VLP Peanut showed strongly reduced vascular leakage of the dye (Fig. 4B).

Passive vaccination with total serum anti-VLP Peanut IgG protects against anaphylaxis

The protective role of allergen specific IgG polyclonal Abs (pAbs)[35] and mAbs[29] against anaphylaxis in a peanut allergy mouse model has been previously demonstrated by our group[29,35]. To investigate whether vaccination with the VLP Peanut vaccine induces IgGs with the same potential, purified total IgGs from pooled sera of BALB/c mice primed and boosted with 50 µg of VLP Peanut were transferred by i.v. injection into peanut sensitized BALB/c mice. The potential for IgGs against anaphylaxis following systemic challenge with Ara R was subsequently assessed (Fig. 5A). To better quantify the protective properties of IgGs induced by VLP Peanut vaccination, a titration of antibodies was performed, ranging from 25 µg/mouse to 100 µg/mouse, with a 25-µg increment. IgGs induced by immunization with $CuMV_{TT}$ (150 µg/mouse) were used as a control. As shown in Fig. 5B, significant protection was achieved in a dose dependent manner for all experimental groups which had received IgGs purified from the sera of mice immunized with VLP Peanut.

The involvement of a low affinity IgG inhibitory receptor FcγRIIb in protection against anaphylaxis was analyzed by next by i.v. injection challenge with peanut extract. To block the receptor function, 150 µg of an anti-FcγRIIb monoclonal antibody (AT 128)[29,35] was administered to animals along with 150 µg of vaccine induced IgGs. As shown in Fig. 5C, protection conferred by a passive transfer of 150 µg IgG induced by VLP Peanut was abrogated by blocking FcγRIIb. Moreover, the mean body temperature drop of a group with inhibited function of FcγRIIb was comparable to that of mice immunized with control $CuMV_{TT}$-specific IgG, confirming that the inhibitory receptor FcγRIIb is mandatory for protection.

Prophylactic vaccination with VLP Peanut protects against future peanut-induced allergic reactions

A prophylactic regimen was tried next to investigate whether the immunization with VLP Peanut of naïve mice prior to sensitization protects against systemic anaphylaxis. Naïve BALB/c mice were first immunized either once or twice (at an interval of 14 days) with 30 µg of VLP Peanut via s.c. route. In addition, the control group was primed and boosted with 30 µg of unmodified

CuMV$_{TT}$. Two weeks after the last vaccine dose had been administered, mice were sensitized to peanut (Fig. 6A). 14 days after sensitization, serum levels of anti-Ara h 2 IgG in mice initially immunized with VLP Peanut were significantly higher than those in sera of control mice (Fig. 6B). Upon systemic challenge with 20 µg of Ara R injected i.v., each group of mice vaccinated with VLP Peanut was protected against anaphylaxis with the same level of significance, with no obvious dose-dependent pattern (Fig. 6C).

To assess the effect of immunization with VLP Peanut as well as sensitization and systemic challenge with Ara R on serum IgE antibody levels, anti-Ara h 2 IgE titers were measured on specific time-points of prophylactic regimen: after immunization, after sensitization and two weeks after systemic challenge. In agreement with results shown above for the therapeutic regimen, no significant changes were detected in anti-Ara h 2 IgE levels measured after sensitization and two weeks after systemic challenge for groups of mice immunized with VLP Peanut. In contrast, specific IgE titers significantly increased following i.v. injection challenge in mice vaccinated with control VLPs (CuMV$_{TT}$). No anti-Ara h 2 IgE specific Abs were detected after immunization prior to sensitization (Fig. 6D).

4. Discussion

A study previously described by our group has shown that vaccine candidates for peanut allergy treatment generated by displaying major peanut allergens (Ara h 1, Ara h 2) or a whole peanut extract (Ara R) to immunologically optimized VLPs (CuMV$_{TT}$) by chemical coupling are highly immunogenic and can confer protection to peanut sensitized mice against local and systemic anaphylaxis[35]. However, the chemical coupling can add complexity to the manufacturing process. For example, two separate bulks of Drug Substance (CuMV$_{TT}$ & Ara h 2) would have to be manufactured and put onto stability trials. A chemical coupling step would also be required, with additional downstream processing to ensure the complete removal of free Ara h 2. While the majority of free (non-coupled) Ara h 2 can be removed via downstream processing, the presence of even trace amounts could have major implications for patient safety.

In the current study, a next-generation, fusion vaccine candidate (VLP Peanut) for the treatment of peanut allergy is described. Subunits of VLP Peanut are expressed within *E. coli* in a single metabolic process and spontaneously assemble to form mosaic VLPs (CuMV$_{TT}$/CuMV$_{TT}$-Ara h 2). The use of a genetic fusion with the polypeptide backbone of VLP subunit simplifies the manufacturing procedure and offers an in-built safety feature excluding contamination with free allergen. Moreover, since a diffusion co-efficient for a sphere is inversely proportional to a size of a sphere and the Ara h 2 present on a surface of VLPs is not evenly distributed in the fluid, the VLP Peanut possesses a lower effective concentration of Ara h 2 than the concertation of “freely dispersed Ara h 2” in a protein extract[37,39]. Therefore, the potential of Ara h 2 displayed on VLPs to interact with and activate mast cells and basophils after s.c. injection is significantly reduced compared to free Ara h 2 and peanut extract. Another important mechanistic perspective is that IgE receptors cannot be cross-linked sufficiently when the allergen is integrated to the VLP-nano-scaffold due to its size and geometry[37,39,40].

The immunization of naïve BALB/c mice with VLP Peanut effectively induced IgG response specific to peanut extract. However, the primary sequences of other 2S albumin peanut allergens Ara h 6 and Ara h 7, share 59% and 42% identity with Ara h 2, respectively[65], what precludes conclusive determination of antibodies specific to Ara h 2 present in peanut extract due to potential cross-reactivity. Because of high prevalence of IgE recognizing Ara h 2 in peanut allergic patients[66], the Ara h 2-specific IgE antibodies are the best serologic marker to diagnose peanut allergy to date. We therefore decided to base our work on induction of specific IgG antibodies against Ara h 2. Immunization of BALB/c mice with VLP Peanut induced significant anti-Ara h 2 IgG responses composed of all IgG subclasses and responses were not significantly different from those induced by Ara h 2 chemically coupled to CuMV$_{TT}$.

Interestingly, IgG responses were enhanced by pre-existing immunity against tetanus toxin (TT), which confirms the potential importance of the introduced universal Th cell epitope derived from TT. The $CuMV_{TT}$ vaccine design is described in Zeltins et al., where T helper cell responses induced by the introduction of the TT epitope have been characterised[43].

The packaging of ssRNA into VLPs during expression in bacterial culture likely plays an essential role in the immunization process. The prokaryotic RNA stabilizes the particle[57,67] and serves as TLR7/8 ligand[68], which promotes IgG class switching[69] and induces the generation of secondary plasma cells[70]. Furthermore, one of the elements of successful specific immunotherapy may be the counter-polarization from "allergic" T_H2 cells towards "anti-allergic" T_H1 cells, reducing allergy-associated imbalanced T cell responses[71]. Indeed, there is preclinical[72-75] and clinical[76,77] evidence that stimulation of TLR7/8 helps during allergen-specific immunotherapy.

Humoral responses in the form of allergen-specific IgG antibodies are considered as one of the elements in development of tolerance/unresponsiveness to allergen exposure. Such IgG protection may be mediated by different mechanisms[23,78]. Allergen bound IgG may physically block the access of IgE to its epitopes on the allergen surface, forming a steric blockade in a manner similar to neutralization of pathogens; hence the name neutralizing or blocking antibodies. In the current study we could clearly demonstrate, that the serum from mice immunized with VLP Peanut effectively inhibits direct binding of high affinity anti-Ara h 2 IgE to its epitope. The affinity of the allergen-specific antibodies is key in this process, as low affinity antibodies fail to neutralize the allergens[28]. Also taking part in the mechanism of action is receptor-dependent mechanism driven by IgG-immune complexes that bind to the inhibitory receptor FcγRIIb. Engaging this receptor by allergen-immune complexes results in a molecular cascade that blocks allergic effector functions of basophils and mast cells[79] and has two important mechanistical implications: 1) in contrast to allergen-neutralization, low affinity IgG antibodies are able to block the allergic response[28,80] and 2) IgG antibodies against a single allergen are be able to block cellular activation by natural, complex allergen mixtures[29,35]. This current study further supports these results.

Subcutaneous immunization of peanut allergic mice with VLP Peanut induced protection against systemic and local anaphylaxis. In addition, repeated systemic challenge confirmed persisting protection from allergy, which is consistent with long-term studies of allergic patients treated with traditional SCIT[81]. Despite this clear protection in comparison to control mice, a slight drop in the body core temperature of mice after i.v. challenge is observable. A significant contribution to this initial temperature drop is due to the standard procedure of lateral tail vein injection in rodents, requiring pre-warming animals to dilate the veins. In addition, and as

opposed to humans, murine neutrophils express Fcγ-receptors able to activate their degranulation[82], causing an anaphylactic reaction. Hence, part of the drop in temperature, seen in particular after the second challenge, may not be caused by allergen-specific IgE but by IgG-allergen immune-complexes rather, activating neutrophils. It should also be emphasized, that the conditions the mice are exposed to under an intravenous challenge with peanut extract are severe and "worst-case". After a repeat challenge scenario, a level of protection was conferred whereby mice did not enter a state of severe anaphylaxis and death, compared to the control group. Thus providing a reassuring proof of principle which confirms the vaccine's protective capacity and its aspirational therapeutic goal.

We have used a classic therapeutic setting to treat peanut allergy. A remaining open question was therefore whether the vaccine induced IgGs were able to prophylactically prevent IgE-mediated anaphylaxis. Integrating allergens within virus-like particles (VLPs) harnesses the properties of a viral structure to stimulate a long-lasting, humoral immune response introducing the concept of prophylactic vaccination into allergy. We have demonstrated the immunogenicity and the cross-protective capacity of a VLP-Ara h2 (VLP Peanut). To test the hypotheses of prophylactic vaccination against peanut allergy, the vaccine was applied in a prophylactic immunization setting in mice. This was assessed in a reversed experiment, where naïve mice were first vaccinated prior to sensitization with peanut extract. Indeed, prophylactic immunization conferred protection against anaphylaxis during systemic challenge. Once immunized with VLP Peanut, natural allergen exposure may maintain protective levels of IgG antibodies against peanut allergy. The results clearly indicate the ability to de-risk systemic anaphylaxis through prophylactic administration of VLP Peanut. Unraveling the preventative potential of AIT in general[83] and our vaccine in particular is an exciting finding opening the field of primary prophylaxis of allergy by VLP-based allergy vaccination.

Important evidence for a protective role of allergen-specific IgGs in allergen immunotherapy has been explored in a phase 1b clinical study[84], where patients suffering from cat allergy were passively treated with two high-affinity, non-overlapping anti-Fel d 1 IgG4 mAbs. The study demonstrated that this passive vaccination reduced allergic symptoms following nasal provocation in these patients. Moreover, the same combination of antibodies administered in a passive cutaneous anaphylaxis (PCA) mouse model showed a protective effect against local mast cell degranulation in a dose dependent manner[83]. Thus, allergen-specific IgG may be able to block the allergic reaction, a finding compatible with earlier observations that an increase in allergen-specific IgG:IgE ratio may be the best correlate of protection in the desensitization process[85,86]. In this current study, peanut sensitized mice were passively immunized with different doses of IgGs induced by VLP Peanut vaccination in naïve mice; up to 100 ug, representing less than 1% of the IgG of the donor mouse[87]. Results demonstrate

that passive immunization with polyclonal anti-Ara h 2 antibodies protect against peanut allergy in a dose dependent manner. In addition, this study broadened the phenomenon of protection against a single allergen to complex mixtures of peanut allergens targeted by anti-Ara h 2 IgGs, thus confirming earlier observations[29,35]. In line with the proposed mode of action, blocking of FcγRIIb function with an anti-FcγRIIb mAb abrogated IgG mediated protection against peanut allergy, highlighting the crucial role of that inhibitory receptor in the mechanism of tolerance induction.

One of the main characteristics and mediators of allergy is a transient increase in systemic IgE levels following allergen exposure[88,89]. Therefore, blocking induction of increased levels of IgE is also a potential therapeutic target for allergy treatment. Indeed, some of the allergen immunotherapies given repetitively for several years may offer disease-modifying effects including inhibition of IgE-dependent allergic reactions[90]. As such, VLP-based vaccine, as described in this manuscript, has a potential to lift classical desensitization offered by existing arsenal of treatments to genuine allergy vaccination. To assess the effect of immunization with VLP Peanut on specific anti-Ara h 2 IgE responses in a peanut allergy mouse model, anti-Ara h 2 IgE titers were measured before, as well as two weeks after, systemic challenge. In contrast to controls, serum levels for specific IgE following the allergen encounter remained unchanged in immunized animals, showing that the stimulation of IgE antibody production can be blocked following allergen exposure, a key asset for long-term treatment and an illustration of non-reactogenicity of the vaccine.

In summary, VLP Peanut ($CuMV_{TT}$/$CuMV_{TT}$-Ara h 2) represents a promising vaccine candidate for the treatment of peanut allergy as it combines greatly reduced allergenicity with strongly increased immunogenicity and pre-clinical efficacy. In addition to our previous study[35], we could show here that the traditional therapeutic treatment of sensitized mice confers protection that is maintained at least for a period of another month after the first challenge. Moreover, the prophylactic regimen of immunization and sensitization is also protective against the future allergen encounter. In both regimens we tested the influence of posology on the protectivity and immunogenicity. In addition, we newly investigated the levels of specific anti-Ara h 2 IgE throughout both regimens. The passive immunization experiment has been performed this time in a dose dependent manner. In parallel to the pre-clinical studies demonstrated in this work, the VLP Peanut manufacturing process was adapted to GMP standards; with the batches manufactured within this process, reproducibility of the presented experiments could be shown. VLP Peanut has now entered clinical development with the initiation of the PROTECT clinical trial (PROTECT trial; ClinicalTrials.gov Identifier: NCT05476497). Nonetheless, because of differences in mouse and human IgG and FcγR biology, the PROTECT clinical trial has a primary and secondary end point of safety measurements. Furthermore, an extensive panel of exploratory biomarkers will be screened as an exploratory proof of efficacy, including

humoral and functional biomarkers. This will also include a panel of Ig subclasses. The functionalities of antibodies are planned to be further studied in subsequent clinical development Phases.

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Figure legends

Fig. 1 Characterization and analysis of VLP Peanut. A, schematic view of the pETDuet-1 plasmid encoding both $CuMV_{TT}$ and $CuMV_{TT}$-Ara h 2 subunit genes, allowing for their co-expression in *E. coli* cells and leading to their co-assembly into mosaic VLPs ($CuMV_{TT}$/$CuMV_{TT}$-Ara h 2); B, TEM image of purified VLP Peanut; C, SDS-PAGE analysis of purified VLP Peanut, M1 - protein molecular weight marker (Marker PageRuler™ Plus Prestained Ladder; 10 to 250 kDa; ThermoFischer Scientific; cat. 26620), asterisk (blue) refers to the subunit ($CuMV_{TT}$) and asterisk (green) refers to the subunit fused to Ara h 2 ($CuMV_{TT}$-Ara h 2); D, native, pre-stained agarose gel analysis of purified VLP Peanut RNA (2), M2 - DNA size marker (GeneRuler 100 bp DNA Ladder; ThermoFischer Scientific; cat. SM0241); E, DLS analysis of purified VLP Peanut.

Fig. 2 VLP Peanut ($CuMV_{TT}$/$CuMV_{TT}$-Ara h 2) induces an anti-Ara h 2 IgG response in naïve BALB/c mice. A, schematic view of vaccination regimen and bleeding schedule; B, $log_{10}OD_{50}$ of anti-Ara h 2 IgG titers from sera of mice vaccinated with $CuMV_{TT}$ as a control and VLP Peanut on days 0, 7, 14, 21 and 28 using D0/D14 immunization regimen; C, $log_{10}OD_{50}$ of the measurements of Ara h 2-specific IgG subclasses serum levels for the group vaccinated with VLP Peanut on days 14 and 28 and $CuMV_{TT}$ on day 28 measured by ELISA; D, competitive ELISA of sera of mice vaccinated with VLP Peanut on days 14 and 28 and $CuMV_{TT}$ on day 28 with anti-Ara h 2 IgE (P12P3D08)[54] of constant concentration 250 ng/mL; VLP Peanut $n = 5$, $CuMV_{TT}$ $n = 5$. Statistical analysis (mean ± SEM) using Mann-Whitney Student's *t*-test for anti-Ara h 2 IgG levels comparison and paired Student's *t*-test for competitive ELISA. The value of $p<0.05$ was considered statistically significant (*$p<0.01$, **$p<0.001$, ***$p<0.0001$).

Fig. 3 VLP Peanut protects against systemic anaphylaxis in a peanut allergy mouse model. A, schematic view of therapeutic regimens used for sensitization and vaccination schedule to assess the efficacy of generated vaccine; B, $log_{10}OD_{50}$ of anti-Ara h 2 IgG titers from sera of sensitized mice vaccinated with $CuMV_{TT}$ as a control and VLP Peanut with a D21/D35 prime/boost schedule; C, $log_{10}OD_{50}$ of anti-Ara h 2 IgG titers from sera collected on day 64 (two weeks after first challenge) from sensitized mice either primed or primed and boosted with VLP Peanut or primed and boosted with $CuMV_{TT}$ as a control; D, E, body temperature courses after i.v. injection challenge of mice followed therapeutic regimen; D, first i.v. injection challenge (day 50); E, second i.v. injection challenge (day 80); F, compared anti-Ara h 2 IgE serum endpoint titers measured along the therapeutic regimen, primed/boosted or only primed with VLP Peanut or primed/boosted with $CuMV_{TT}$ as control, measured by ELISA with *Cutoff* = 0.13. Primed/boosted VLP Peanut *n = 5*, primed VLP Peanut *n = 5*, primed/boosted $CuMV_{TT}$ *n = 5*. Statistical analysis (mean ± SEM) using Mann-Whitney Student's *t*-test for anti-Ara h 2 IgG levels comparison, Student's *t*-test with Welch's correction for body temperature measurements as well as Welch and Brown-Forsythe ANOVA for anti-Ara h 2 IgE levels comparison. The value of $p<0.05$ was considered statistically significant (*$p<0.01$, **$p<0.001$, ***$p<0.0001$).

Fig. 4 Effects of VLP Peanut when used in a skin prick test (SPT). A, schematic view of the sensitization and vaccination regimen used to perform SPT; B, area of the surface of the Evans Blue dye extravasation into the ear tissue quantified after ears collection using Fiji ImageJ software. VLP Peanut *n = 5*, $CuMV_{TT}$ *n = 5.* Statistical analysis (mean ± SEM) using Student's *t*-test with Welch's correction. The value of $p<0.05$ was considered statistically significant (*$p<0.01$, **$p<0.001$, ***$p<0.0001$).

Fig. 5 Passive transfer of IgG induced by VLP Peanut protects against anaphylaxis. A, schematic view of immunization regimen for IgG induction, purification, passive transfer to peanut-sensitized mice and i.v. injection challenge; B, body temperature course following i.v. injection challenge of peanut-sensitized mice passively vaccinated with 25, 50, 75 or 100 µg of IgG induced by VLP Peanut vaccination or 150 µg of IgG induced by $CuMV_{TT}$ vaccination as control; C, body temperature course following i.v. challenge of peanut-sensitized mice passively vaccinated with 150 µg of IgG induced VLP Peanut with or without 150 µg of anti-FcγRIIb mAbs or 150 µg of IgG induced by $CuMV_{TT}$ as control. Each group *n = 4*. Statistical analysis (mean ± SEM) using Student's *t*-test with Welch's correction. The value of $p<0.05$ was considered statistically significant (*$p<0.01$, **$p<0.001$, ***$p<0.0001$).

Fig. 6 VLP Peanut protects against systemic anaphylaxis when used in a prophylactic immunization regimen. A, schematic view of prophylactic regimens used for vaccination and sensitization schedule to assess the efficacy of generated vaccine; B, $log_{10}OD_{50}$ of anti-Ara h 2 IgG titers from sera of mice followed prophylactic regimen 2 weeks after sensitization (D49 for mice with a prime/boost vaccination schedule and D35 for mice with prime vaccination schedule); C, body temperature courses of mice followed prophylactic regimen after i.v. injection challenge (D50 for mice with a prime/boost vaccination schedule and D36 for mice with prime vaccination schedule); D, compared anti-Ara h 2 IgE serum endpoint titers measured along the prophylactic regimen, primed/boosted or only primed with VLP Peanut or primed/boosted with $CuMV_{TT}$ as control, measured by ELISA with *Cutoff* = 0.13. Primed/boosted VLP Peanut *n = 5*, primed VLP Peanut *n=5*, primed/boosted $CuMV_{TT}$ *n = 5*. Statistical analysis (mean ± SEM) using Mann-Whitney Student's *t*-test for IgG levels comparison, Student's *t*-test with Welch's correction for body temperature measurements, Welch and Brown-Forsythe ANOVA for IgE levels comparison. The value of $p<0.05$ was considered statistically significant (*$p<0.01$, **$p<0.001$, ***$p<0.0001$).

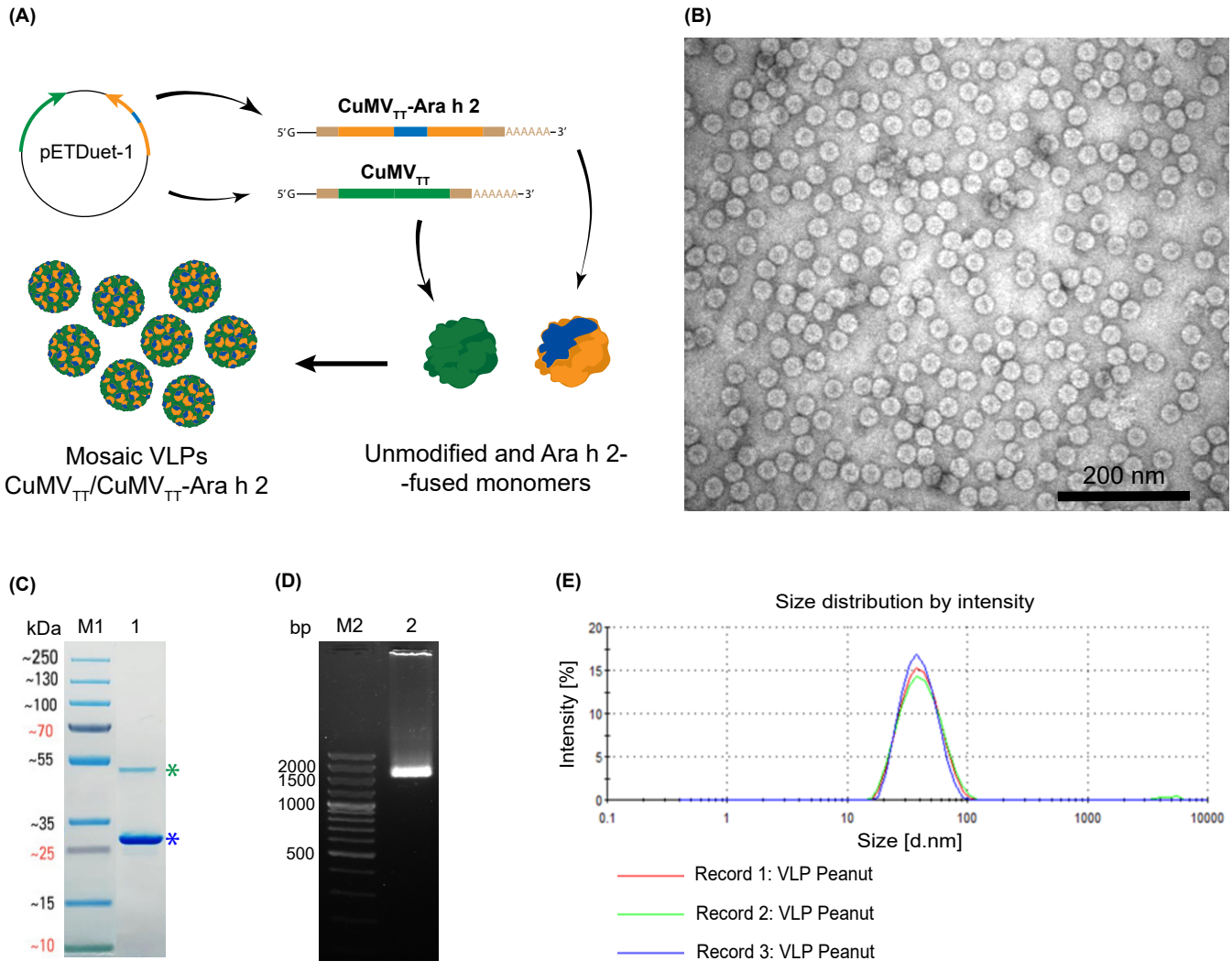


Figure 1_Sobczak et al.

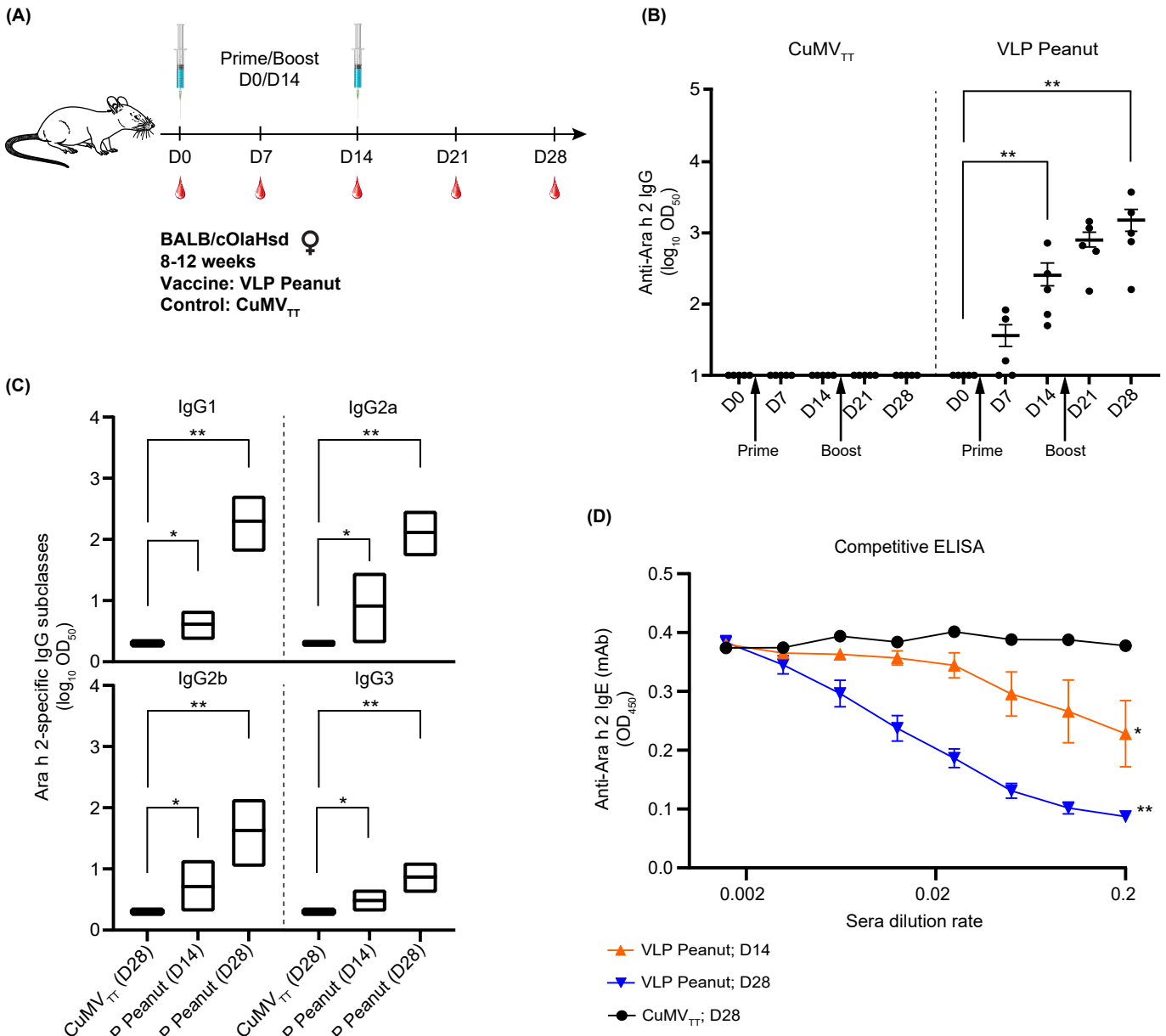
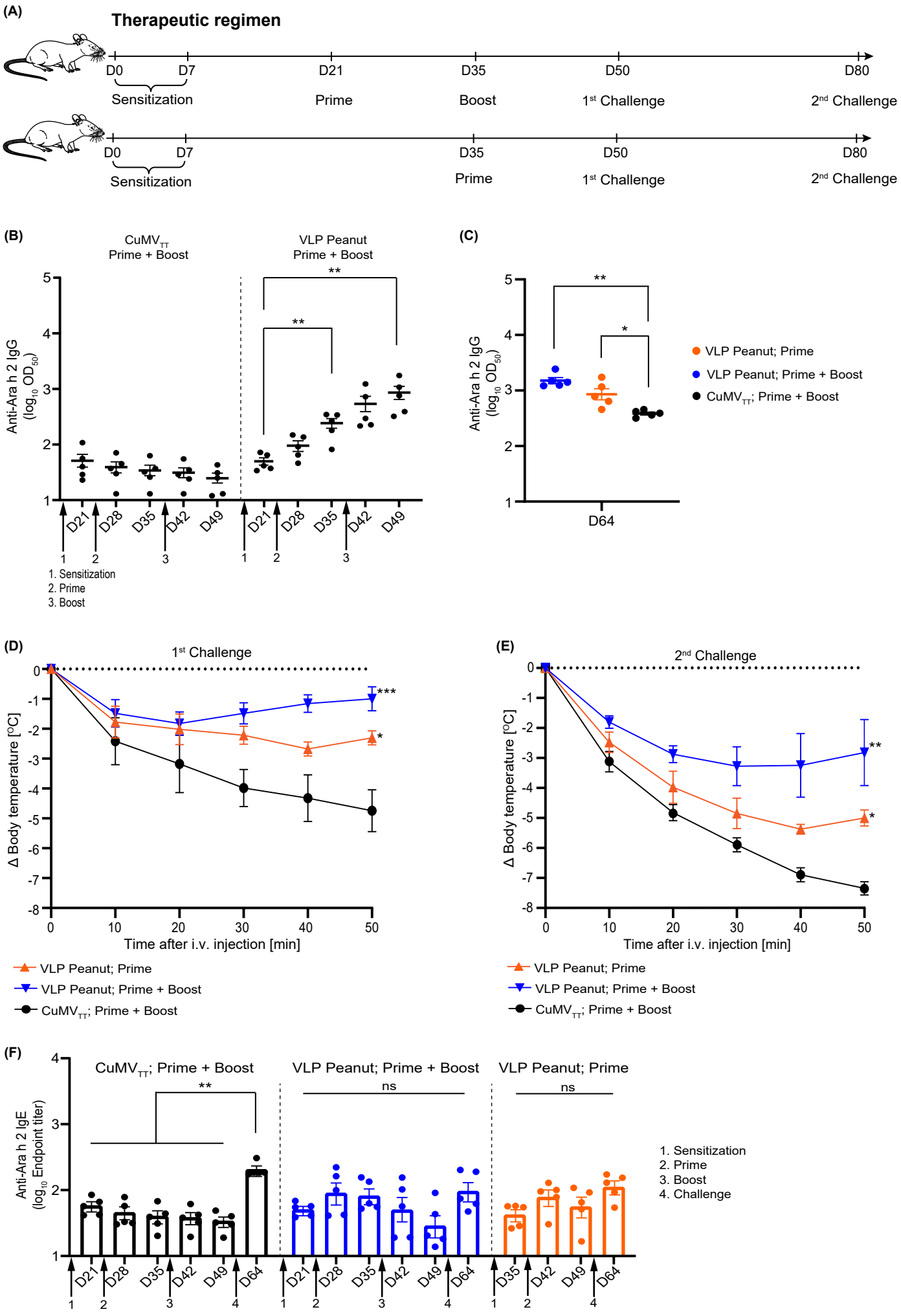


Figure 2_Sobczak et al.



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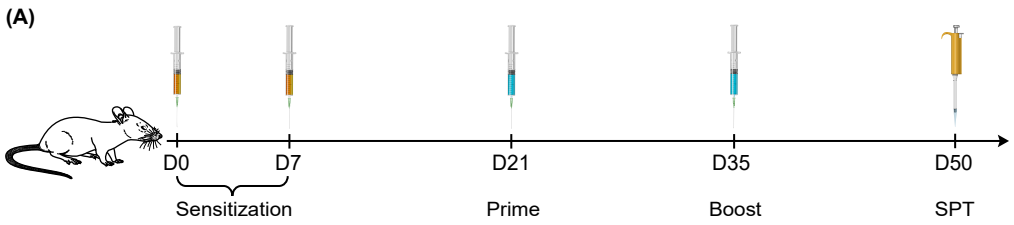

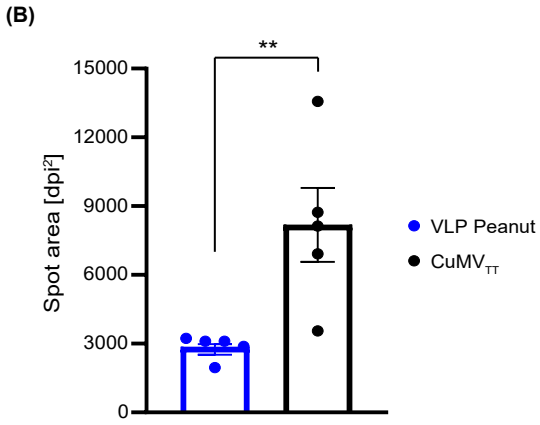

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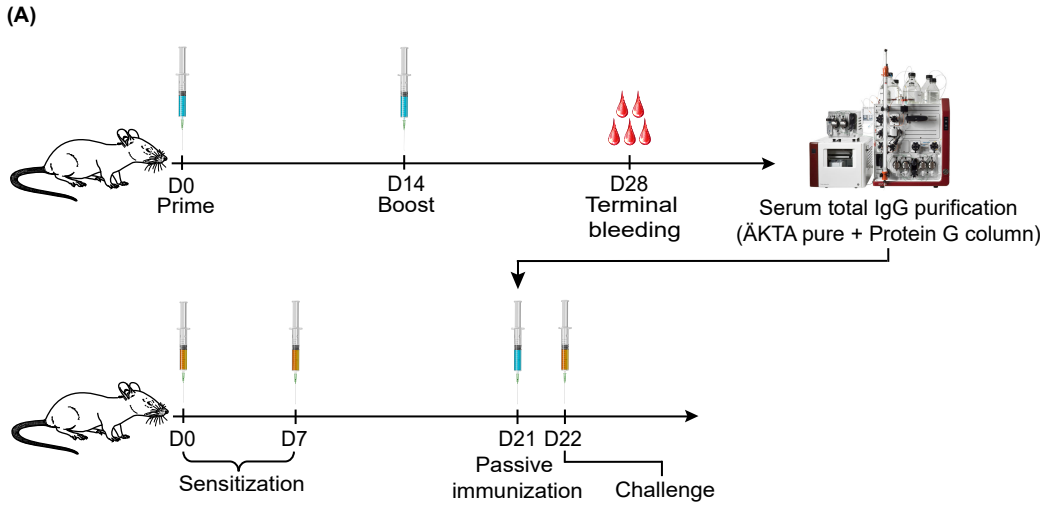

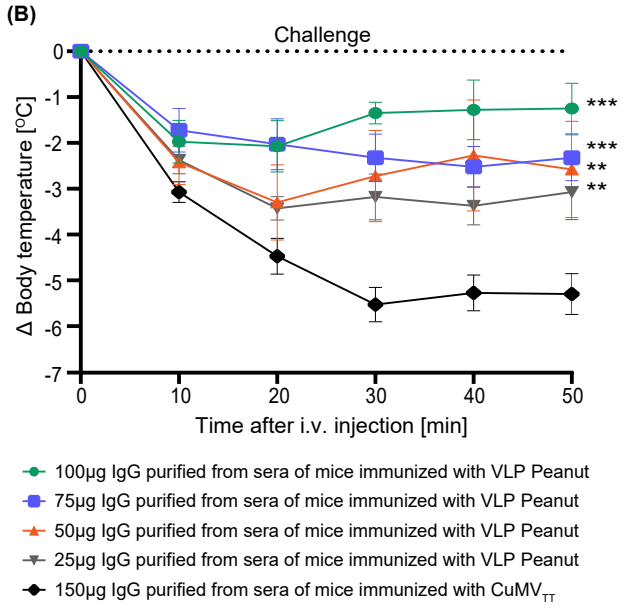

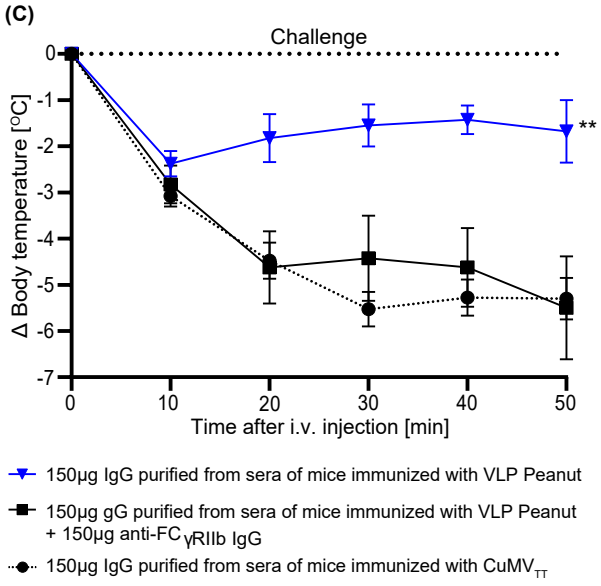

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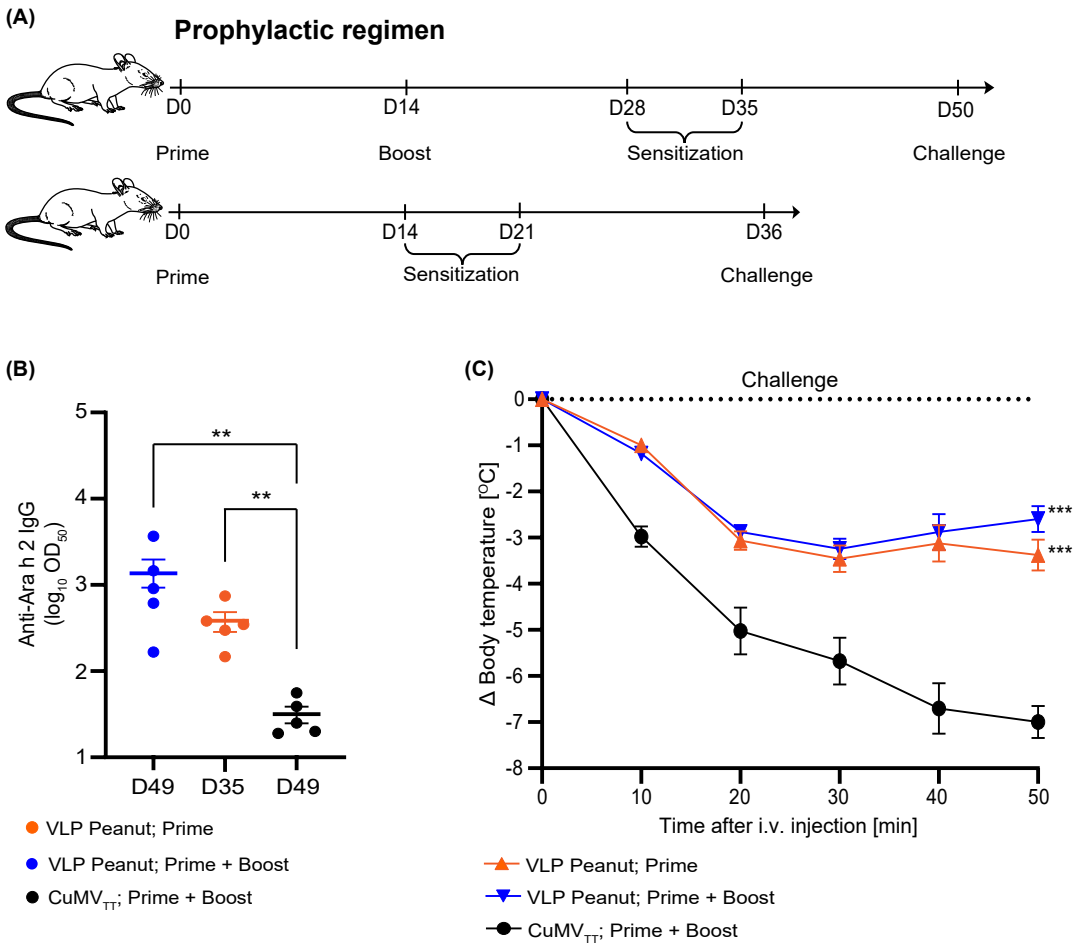

Figure 6_Sobczak et al.