

REIVINDICACIONES

1. Una partícula tipo virus modificada (VLP) del virus del mosaico del pepino (CMV), que comprende una pluralidad de polipéptidos de CMV modificados, en donde cada dicho polipéptido de CMV modificado comprende
 - (a) un polipéptido de CMV, donde dicho polipéptido de CMV comprende una secuencia de aminoácidos de una proteína de recubrimiento de CMV que comprende la SEQ ID NO. 1 o SEQ ID NO. 21; y
 - (b) un epítipo de célula T ayudante, en donde dicho epítipo de célula T ayudante es seleccionado de cualquiera de las secuencias SEQ ID NO:4, SEQ ID NO: 5 o SEQ ID NO: 26 a 30 y en donde dicho epítipo de célula T ayudante sustituye una región N-terminal de dicho polipéptido de CMV que corresponde a los aminoácidos 2 a 12 de la SEQ ID NO. 1.
2. La VLP modificada de CMV de conformidad con la Reivindicación 1, en donde dicho polipéptido de CMV comprende una secuencia de aminoácidos de la SEQ ID NO:6.
3. La VLP modificada de CMV de conformidad con la Reivindicación 1, en donde dicho polipéptido de CMV comprende una secuencia de aminoácidos de la SEQ ID NO:7.
4. Una composición que comprende una partícula tipo virus modificada de conformidad con cualquiera de las Reivindicaciones 1 a 3.
5. La composición de conformidad con la Reivindicación 4, en donde dicha composición comprende:
 - (a) una partícula tipo virus modificada de conformidad con cualquiera de las reivindicaciones 1 a 3, y en donde dicha partícula tipo virus modificada comprende uno o más primer sitio de unión; y
 - (b) al menos un antígeno, en donde dicho antígeno comprende uno o más segundo sitio de unión;en donde (a) y (b) están vinculados a través de dicho uno o más primer y dicho uno o más segundo sitio de unión a través de por lo menos un enlace no peptídico covalente.
6. La composición de conformidad con la Reivindicación 5, en donde dicho primer sitio de unión es un grupo amino.

7. La composición de conformidad con cualquiera de las reivindicaciones 5 a 6, en donde dicho primer sitio de unión es un grupo amino de un residuo de lisina.
8. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 7 en donde dicho segundo sitio de unión es un grupo sulfhidrilo.
9. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 7, en donde dicho segundo sitio de unión es un grupo sulfhidrilo de un residuo de cisteína.
10. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 9, en donde dicho primer sitio de unión no es un grupo sulfhidrilo.
11. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 10, en donde sólo uno de dichos segundos sitios de unión se asocia con dicho primer sitio de unión a través de uno o más enlaces covalentes no peptídicos que conducen a un tipo único y uniforme de unión de dicho antígeno a dicha partícula tipo virus modificada, en donde dicho sólo un segundo sitio de unión que se asocia con dicho primer sitio de unión es un grupo sulfhidrilo, y en donde dicho antígeno y dichas partículas tipo virus modificadas interactúan a través de dicha asociación para formar una matriz antigénica ordenada y repetitiva.
12. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 11, en donde dicho segundo sitio de unión es un grupo sulfhidrilo unido mediante acoplamiento químico.
13. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 12 que además comprende un vinculador, en donde dicho vinculador asocia dicho antígeno con dicho segundo sitio de unión.
14. La composición de conformidad con la Reivindicación 13, en donde dicho vinculador comprende dicho segundo sitio de unión.
15. La composición de conformidad con cualquiera de las Reivindicaciones 4 a 14, que además comprende una o más sustancias inmunoestimulantes, en donde dicha sustancia inmunoestimulante se une a, se mezcla con o se empaca en dichas partículas tipo virus.
16. La composición de conformidad con la Reivindicación 15, en donde dicha sustancia inmunoestimulante se empaca en dicha partícula tipo virus modificada.

17. La composición de conformidad con la Reivindicación 15 o 16, en donde dicha sustancia inmunoestimulante consiste de ADN o ARN de origen no eucariota.
18. La composición de conformidad con cualquiera de las Reivindicaciones 15 a 17, en donde dicha sustancia inmunestimulantes es un oligonucleótido que contiene CpG no metilado.
19. La composición de conformidad con la Reivindicación 18, y en donde dicho oligonucleótido que contiene CpG no metilado comprende una secuencia palindrómica.
20. La composición de conformidad con la Reivindicación 18 o 19, y en donde el motivo de CpG de dicho oligonucleótido que contiene CpG no metilado es parte de una secuencia palindrómica.
21. La composición de conformidad con la Reivindicación 19 o 20, en donde dicha secuencia palindrómica es GACGATCGTC (SEQ ID NO: 31).
22. La composición de conformidad con cualquiera de las Reivindicaciones 19 a 21, en donde dicha secuencia palindrómica es flanqueada en su 5'-terminal por de 3 a 5 entidades de guanosina, y en donde dicha secuencia palindrómica es flanqueada en su 3'-terminal por de 3 a 5 entidades de guanosina.
23. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es un antígeno de tumor, un antígeno propio, un polipéptido de un patógeno, un alérgeno o un hapteno.
24. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 23, en donde dicho antígeno es IL-17.
25. La composición de conformidad con la Reivindicación 24, en donde dicho antígeno comprende la SEQ ID NO: 42.
26. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es IL-5.
27. La composición de conformidad con la Reivindicación 26, en donde dicho antígeno comprende la SEQ ID NO: 41.

28. La composición de conformidad con cualquiera de las Reivindicaciones de 5 a 22, en donde dicho antígeno es TNF α .
29. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es IL-1 α .
30. La composición de conformidad con cualquiera de las Reivindicaciones de 5 a 22, en donde dicho antígeno IL-13.
31. La composición de conformidad con la Reivindicación 30, en donde dicho antígeno comprende la SEQ ID NO: 46.
32. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es un péptido derivado de A1-42.
33. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es IgE o un péptido o dominio comprendido en IgE.
34. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es α -sinucleína o un péptido derivado de α -sinucleína.
35. La composición de conformidad con la Reivindicación 34, en donde dicho antígeno es un péptido derivado de α -sinucleína que consiste en 6 a 14 aminoácidos.
36. La composición de conformidad con la Reivindicación 34 o 35, en donde dicho antígeno es un péptido derivado de α -sinucleína seleccionado de cualquiera de las secuencias SEQ D NO:32, SEQ ID NO:33 y SEQ ID NO:34.
37. La composición de conformidad con cualquiera de las Reivindicaciones de 5 a 22, en donde dicho antígeno IL-4.
38. La composición de conformidad con la Reivindicación 37, en donde dicho antígeno comprende la SEQ ID NO: 45.
39. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es GnRH.

40. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es eotaxina.
41. La composición de conformidad con cualquiera de las Reivindicaciones de 5 a 22, en donde dicho antígeno es el dominio extracelular de la proteína M2 del virus de la Influenza A, un fragmento antigénico del mismo.
42. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es IL-31.
43. La composición de conformidad con la Reivindicación 42, en donde dicho antígeno comprende las SEQ ID NO:43 o SEQ ID NO: 44.
44. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno se deriva de Plasmodium falciparum o Plasmodium Vivax, o en donde dicho antígeno es el derivado de RSV.
45. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es el alérgeno de gato Fel d1 o un Fel d1 recombinante (rFel d1).
46. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es un Fel d1 recombinante (rFel d1).
47. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno comprende la SEQ ID NO: 17 o SEQ ID NO: 18.
48. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno consiste de la SEQ ID NO: 17 o SEQ ID NO: 18.
49. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno comprende la SEQ ID NO: 18.
50. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es el alérgeno de perro Can f1 o Can f2.
51. La composición de conformidad con cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es el péptido relacionado con el gen calcitonina (CGRP).

52. La composición de cualquiera de las Reivindicaciones 5 a 22, en donde dicho antígeno es Amilina.

53. Una vacuna que comprende la partícula tipo virus modificada de cualquiera de las Reivindicaciones 1 a 3 o la composición de cada una de las Reivindicaciones 4 a 52.

54. Una composición farmacéutica que comprende:

- (a) la partícula tipo virus modificada de conformidad con cualquiera de las Reivindicaciones 1 a 3, la composición de cualquiera de las Reivindicaciones 4 a 52, o la vacuna de la Reivindicación 53; y
- (b) un portador, diluyente y/o excipiente farmacéuticamente aceptable.

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ARTICLE OPEN

Incorporation of tetanus-epitope into virus-like particles achieves vaccine responses even in older recipients in models of psoriasis, Alzheimer's and cat allergy

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Monoclonal antibodies are widely used to treat non-infectious conditions but are costly. Vaccines could offer a cost-effective alternative but have been limited by sub-optimal T-cell stimulation and/or weak vaccine responses in recipients, for example, in elderly patients. We have previously shown that the repetitive structure of virus-like-particles (VLPs) can effectively bypass self-tolerance in therapeutic vaccines. Their efficacy could be increased even further by the incorporation of an epitope stimulating T cell help. However, the self-assembly and stability of VLPs from envelope monomer proteins is sensitive to geometry, rendering the incorporation of foreign epitopes difficult. We here show that it is possible to engineer VLPs derived from a non human-pathogenic plant virus to incorporate a powerful T-cell-stimulatory epitope derived from Tetanus toxoid. These VLPs (termed CMV_{TT}) retain self-assembly as well as long-term stability. Since Th cell memory to Tetanus is near universal in humans, CMV_{TT}-based vaccines can deliver robust antibody-responses even under limiting conditions. By way of proof of concept, we tested a range of such vaccines against chronic inflammatory conditions (model: psoriasis, antigen: interleukin-17), neurodegenerative (Alzheimer's, β -amyloid), and allergic disease (cat allergy, Fel-d1), respectively. Vaccine responses were uniformly strong, selective, efficient *in vivo*, observed even in old mice, and employing low vaccine doses. In addition, randomly ascertained human blood cells were reactive to CMV_{TT}-VLPs, confirming recognition of the incorporated Tetanus epitope. The CMV_{TT}-VLP platform is adaptable to almost any antigen and its features and performance are ideally suited for the design of vaccines delivering enhanced responsiveness in aging populations.

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INTRODUCTION

Vaccines are widely used prophylactically to prevent infectious disease, as well as therapeutically to alter the course of established chronic infections. While passive immunization in the form of monoclonal antibodies ("biologics") has had a dramatic impact on non-infectious diseases, including cancer, chronic inflammatory, and neurodegenerative disease, the development of vaccines in these increasingly important therapeutical areas has only very recently been explored clinically. The term 'therapeutic vaccine' has been used to denote vaccines aiming at blocking an endogenous molecular pathway in order to alter the course of an already established non-infectious condition. This approach represents a potentially attractive public health option as it could offer an affordable alternative to several mAb therapies (chapter 54 in ref. 1). Examples include vaccines against angiotensin to treat hypertension,^{2,3} TNF α to treat arthritis,⁴ IFN α to treat lupus,⁵ or IL-1 β to treat diabetes.⁶ Such vaccines have been designed using a variety of strategies, including DNA-vaccination, coupling to non-specific adjuvants such as keyhole limpet hemocyanin (KLH), or virus-like particles (VLP, reviewed in⁷). However, inconsistent neutralizing capacity of vaccine-induced target-

specific antibodies has often been found limiting. This may be due to a combination of factors, including the absence of good Th cell epitopes within the vaccine conferring good Th cell help to B cells,^{8–10} difficulties to bypass self-tolerance, as well as poor vaccine responses in an aging demographic due to immunosenescence.

Regarding bypassing of self-tolerance we have previously shown that the use of VLPs as antigen-carrier confers robust B cell activation, by mimicking the repetitive three-dimensional scaffold common to viral intruders.^{7,11,12} Building on this work, we here sought to explore whether incorporation of a strong T-cell epitope might deliver enhanced T cell help to B cells even under limiting conditions, such as aged vaccine recipients. The T-cell epitope derived from the tetanus toxin (TT) is uniquely capable of delivering such intrinsic immuno-boosting, since pre-existing T cell memory to this epitope is near universal in humans and has been used as such in vaccines.^{13,14} However, internal genetic fusion of this epitope to a viral envelope protein has not been reported and is not trivial since the capacity for self-assembly of icosahedral particles from VLP monomer proteins is highly sensitive to altered geometry. The search for suitable parent virus

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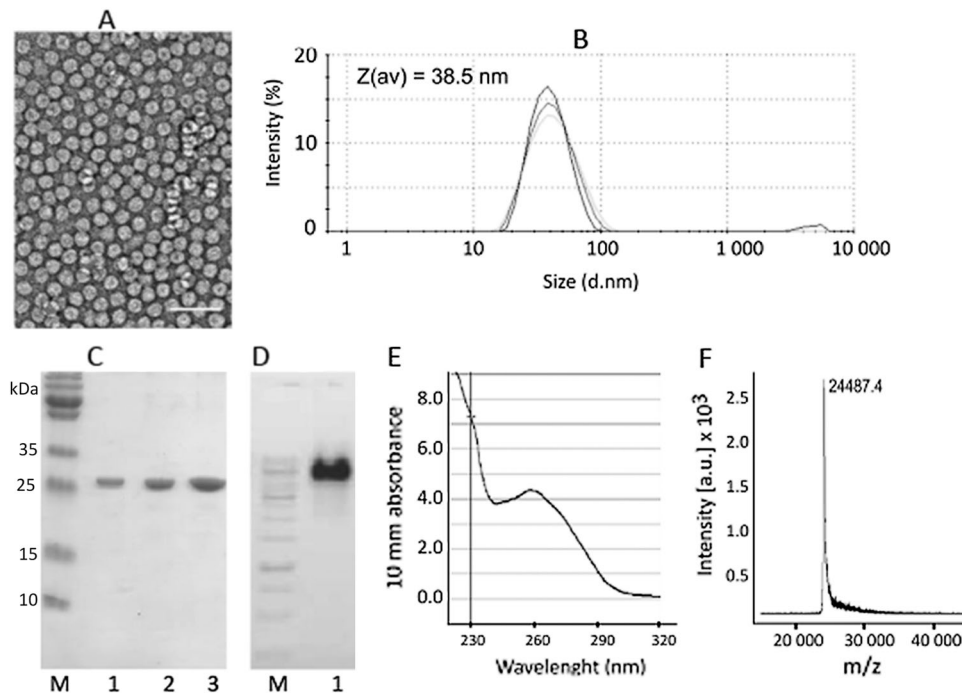


Fig. 1 Properties of CMV_{TT} virus-like particles. **a** Electron microscopy image of purified VLPs incorporating the universal T-cell epitope (Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu) derived from Tetanus toxin. VLP sample (1.5 mg/ml) was adsorbed on carbon formvar-coated copper grids and were negatively stained with 1% uranyl acetate aqueous solution. The grids were examined using a JEM-100C electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV. White bar corresponds to 100 nm. Particle size was found between 26 and 28 nm. **b** Dynamic light scattering (DLS) analysis of CMV_{TT}. Sample VLP solution (1 mg/ml) was analyzed on a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, UK). The results of three measurements were analyzed by DTS software (Malvern, version 6.32). The average hydrodynamic diameter (Z(av)) of particles was found 38.5 nm. **c** SDS-PAGE analysis of CMV_{TT} VLPs. Increasing amounts of VLPs were loaded on the gel (lane 1–0.6 µg, lane 2–1.2 µg and lane 3–2.4 µg). M—protein size marker (Page Ruler Plus, Thermo Scientific). Samples were derived from the same experiment and were processed in parallel. **d** Agarose gel analysis of CMV_{TT}. Lane 1 – CMV VLPs (4 µl, 1.5 mg/ml) were mixed with DNA Loading buffer (lane 1) and analyzed in 0.8% agarose /TBE buffer. M – DNA size marker (Gene Ruler, 1 kb, Thermo Scientific). Samples were derived from the same experiment and were processed in parallel. **e** UV spectroscopy. The UV spectrum of VLP solution (1 mg/ml) was recorded using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The VLP sample absorbs strongly at 260 nm which is typical for viruses and VLPs. **f** For mass spectrometric (MS) analysis CMV_{TT} VLPs (1 mg/ml) were diluted with a 3-hydroxypicolinic acid matrix solution and were spotted onto an MTP AnchorChip 400/384TF. Matrix-assisted laser desorption/ionization (MALDI)-TOF MS analysis was carried out on an Autoflex MS (Bruker Daltonik, Germany). The protein molecular mass (MM) calibration standard II (22.3–66.5 kDa; Bruker Daltonik) was used for mass determination. Obtained spectrum suggests that the first methionine is removed during the CMV coat protein synthesis in *E. coli* cells and the N-terminus is replaced by the TT epitope (calculated MW 24485 Da, found *m/z* value 24487.3 Da)

shells is further complicated by additional constraints required to obtain a platform adaptable to clinical applications including the avoidance of human pathogenic virus species, as this may perturb classical vaccination programs or interfere with diagnostics. Furthermore, selection of a sufficiently large VLP-monomer protein would increase the overall number of additional potential Th cell epitopes derived from the VLP itself.

Here we demonstrate that VLPs derived from Cucumber Mosaic Virus can be engineered to incorporate an internally fused Tet-epitope. Vaccines based on the resultant VLPs (CMV_{TT}) are stable, immunogenic, and elicit strong responses even under limiting conditions. In terms of safety of plant virus-derived VLP, a recent clinical trial employing a plant-derived VLP-vaccine to treat malaria did not identify limiting safety aspects.¹⁵ Cucumber mosaic virus is a widely distributed virus with no toxicity to humans reported to date. In addition, there are several studies using CMV-derived vaccines in mammals including pigs, without identifying toxicity.¹⁶ CMV_{TT} can easily be linked to any antigen of choice via chemical cross-linking. As such, this new technology should significantly increase the utility of vaccine approaches both for the efficient targeting of signaling pathways in vivo, as well as for other clinical applications.

RESULTS AND DISCUSSION

Construction of a stable VLP containing a universal T cell epitope We set out to engineer a VLP particle maintaining structural stability as well as the capacity for self-assembly even after incorporation of an artificial sequence into the envelope protein monomers. In addition, in order to be adaptable to a wide range of applications, the ideal VLP would (1) be derived from a non-human parent virus, (2) retain the capacity to incorporate RNA able to serve as immuno-stimulatory TLR agonist RNA,^{17,18} and (3) exhibit a large molecular size monomer to increase the overall number of T-cell stimulatory epitopes. Based on these criteria, we screened several candidate plant virus derived VLPs harboring large (25–30 kDa) monomer blocks, almost twice the molecular weight of e.g., RNA-phage derived VLPs. Several candidate VLPs exhibited dramatically reduced or absent VLP stability upon insertion of the Tetanus-derived epitope (TT) at various sites (Suppl. Fig. 1). In contrast, replacement of the first 12 N-terminal amino acids of CMV with the TT epitope resulted in well assembled VLPs with a size of approximately 30–40 nm in diameter, as shown by electron microscopy (EM) (Fig. 1a) and dynamic light scattering (DLS) (Fig. 1b), preserving the native T = 3 icosahedral structure. SDS-PAGE analysis revealed a homogenous product of the expected size (Fig. 1c). Ethidium bromide staining

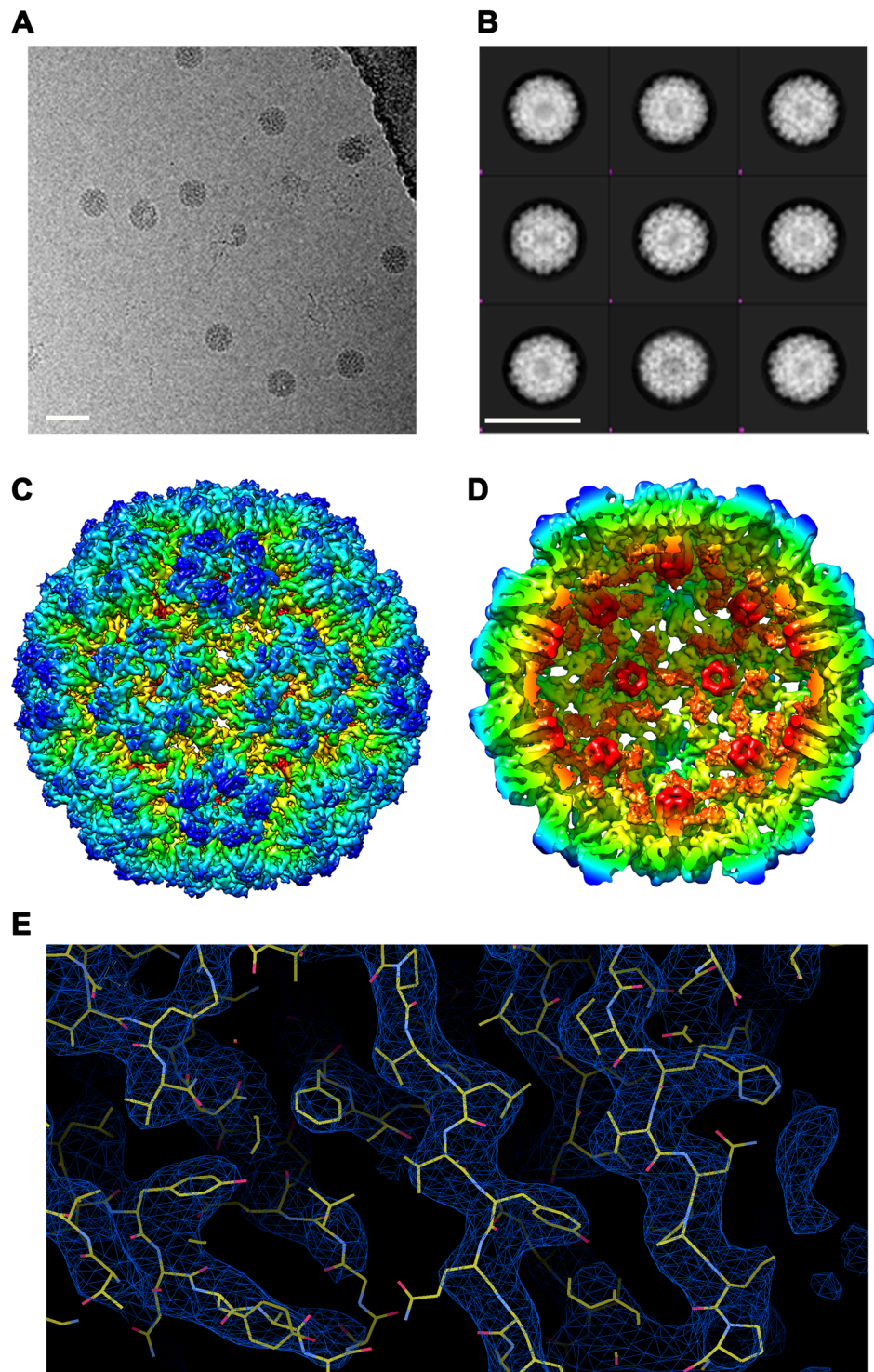


Fig. 2 Structure resolution of engineered CMV VLPs by cryoEM. VLPs were dissolved in borate-EDTA containing buffer, vitrified and analyzed by EM using a Tecnai F30 'Polara' microscope (FEI) **a** Representative 2-D view of vitrified VLP, generated from repeated imaging each field of view along the Z- axis creating a series of images (constituting a movie). From ~300 movies, 6685 particles were selected for data processing. **b** Examples of generated 2-D classification, 20 were generated based on average electron density. White bar corresponds to 50 nm. Cross sectional Selection refinement towards 3-D electron density model and an initial model fitting data to icosahedral symmetry produced 9 Å model. **c** High level refinement further improved resolution to 4.2 Å. In addition, **d** the internal view reveals ordered elements (shown in orange) are visible on the interior of the capsid shell. **e** Resolution Fitting reconstruction reveals that the obtained structure corresponds to X-ray crystal structural data

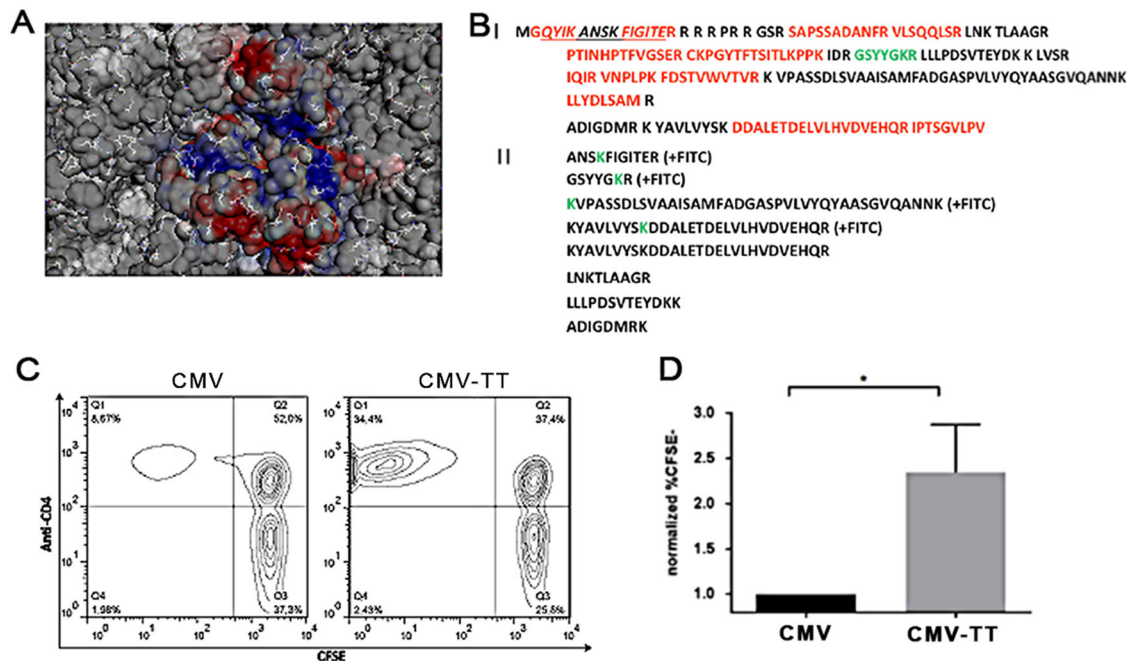


Fig. 3 Chemical reactivity of surface exposed Lysine residues and T cell reactivity of incorporated universal T cell epitope. **a** Surface representation of cryoEM model with all lysine residues displayed in stick format, net surface charge is color coded. **b** Trypsin fingerprint analysis of CMV-Ntt830 VLPs after treatment with fluorescein isothiocyanate (FITC). **Panel I**—CMV-Ntt830 protein sequence represented in the form of tryptic peptides. Red—peptides identified after trypsin digest and mass spectrometric analysis; black—peptides not found; green—peptide identified only in the form of FITC conjugate. The Ntt830 residues are underlined; **Panel II**—CMV-Ntt830 coat protein peptides found as FITC conjugates (green Lys symbols) or partially cut by trypsin. **c, d** The universal T cell epitope in CMV_{TT} is recognized by primary human CD4⁺ T cells. **c** Human PBMCs from four individual donors were labeled with CFSE and cultured for 7 days upon stimulation with either CMV or CMV_{TT} and CFSE fluorescence was assessed by flow cytometry. Shown are representative scatterplots of CD4⁺ T cells stimulated with either CMV or CMV_{TT}. **d** Normalized percentages of CFSE-CD4⁺ T cells in 4 individual donors upon CMV (black) or CMV_{TT} (gray) are displayed as mean $\pm$ SEM. * $p < 0.01$ in a two-tailed, unpaired student's t test

(Fig. 1d) and UV spectroscopy (Fig. 1e) confirmed preserved RNA incorporation. Mass spectroscopy confirmed one major peak of the expected size (Fig. 1f). CMV_{TT}-VLPs were stable for at least 12 weeks both at -20°C or 4°C (Suppl. Fig. 2). This was further confirmed by DLS analysis showing stability with only a low tendency to aggregate generation as indicated by a second peak with increased mean hydrodynamic size (Z (av)).

Structure of CMV_{TT} VLPs

We next characterized the molecular structure of the newly generated CMV_{TT} VLPs by cryoEM. High resolution images were captured (Fig. 2a) and more than 6600 VLPs were picked for averaging, resulting in several 2D classes of VLPs (Fig. 2b); the best of these were used for 3D- classification. This allowed construction of a $9\text{ \AA}$ model, followed by refinement to $4.2\text{ \AA}$ (Figs. 2c, d), and fitting of the primary sequence into the structure (Fig. 2e). The structure of CMV_{TT} appeared largely identical to the parent CMV particles (RMSD $1.8\text{ \AA}$), particularly at the surface. Specifically, the subunit arrangement of the asymmetric unit (chains A, B and C) was identical between the parent virus and the modified VLPs. The AA dimers were arranged at the 5-fold axis and the BC dimers around the 3-fold/ pseudo 6-fold axis. Upon closer inspection there were minor differences in the spatial location of the backbone polypeptide in the capsid interior, most likely due to the lack of native viral capsid-genome interactions in the recombinant VLPs. Instead, the modified VLP exhibited additional electron density displaying 3-fold symmetry within the interior cavity (Fig. 2d, orange color), indicative of novel ordered structures, most likely reflecting tightly bound internal RNA. Interestingly, their relative location and resemblance to tightly-associated RNA species would suggest they might contribute towards stabilizing

of intra-subunit associations underlying the overall stability of the particles. A further intriguing aspect of the structure is the relatively large pore size, which may allow for the exchange of naturally bound RNA by other poly-anions or other cargo.

A straightforward method to display antigens of choice on the surface of VLPs is through chemical coupling to surface exposed Lys. We therefore mapped all surface exposed Lys in the VLP-structure (Fig. 3a). Next, FITC conjugation to CMV_{TT}, followed by trypsin-digest and MS was used to identify Lys residues functionally available for coupling. This showed that 4 of 8 tryptic peptides generated were detected with the FITC modification (Fig. 3b). One surface located Lys-containing peptide (GSYYGKR, green letters in Fig. 3b, Panel I) was completely modified by FITC. In all other cases, both unmodified and FITC-modified peptide forms were found. These results correspond well to the cryoEM structural model, which shows several surface located Lys to be orientated inwards thereby providing insufficient space for multiple crosslinker-VLP interactions. Ionic charge also was an efficient predictor of coupling, since two prominent surface exposed Lys (equivalent to K79 and K116 in the wild type virus) located in acidic patches did not couple to FITC, while K88 residing nearby but with different charge was efficiently modified. Taken together, these data provide an in-depth structural characterization of the novel VLP-species and confirm that ample surface-exposed lysine residues are present to allow chemical cross-linking to antigenic epitopes of choice.

Recognition of the universal T cell epitope in CMV_{TT} by human CD4⁺ T cells

In order to test recognition of the tetanus-derived universal T cell epitope, human PBMCs were labeled with CFSE and stimulated

with either wild type or CMV_{TT} followed by measurement of proliferation by assessment of the frequency of CFSE^{low} CD4⁺ T cells 7 days later. With each cell division, cellular CFSE-labeling is reduced by 50% and CFSE^{low} CD4⁺ T cells therefore represent T cells having undergone cell division. The frequency of these cells was much higher in PBMC cultures stimulated with CMV_{TT} compared to those stimulated with CMV wild type, in fact comparable to proliferation of cells stimulated with pure tetanus toxoid as positive control, thereby confirming robust recognition of the universal T cell epitope in CMV_{TT} (Fig. 3c, left upper quadrant and Fig. 3d). Therefore, incorporation of the universal T-epitope in CMV_{TT} boosts T-cell responses in randomly selected primary human T cells.

Generation of a vaccine against psoriasis

We next proceeded to design clinically relevant vaccine prototypes based on CMV_{TT} to explore functional performance. First, we focussed on Interleukin 17 A (IL17A), a pro-inflammatory cytokine. Antibodies targeting IL17A are highly effective in psoriasis, a skin condition affecting between 1 and 4 % in most global populations.¹⁹ Despite an excellent safety and efficacy profile, the high cost of IL17-targeting biologics severely restricts access to this treatment. Replacing these antibodies by vaccination against IL-17A therefore has the potential to vastly increase access to treatment for a wide range of patients (reviewed in ref. 20) as well as be useful for other conditions, as we have previously shown using an earlier VLP-based IL17 vaccine.²¹ To test the performance of a CMV_{TT} based anti-IL17A vaccine for psoriasis, we first generated a vaccine prototype by conjugation of full-length, dimeric murine IL-17A to CMV_{TT} (Figure S3). Next, mice were immunized with different doses of the IL-17 CMV_{TT} vaccine preparation, resulting in high anti-IL-17A IgG levels, after as low a dose as 0.5 µg (Fig. 4a), but not to the most highly conserved IL17 isoform, IL-17F (Fig. 4b), confirming high selectivity of the induced response.

Preclinical efficacy of the IL17CMV_{TT}

We next assessed the ability of the vaccine to reduce psoriatic disease in vivo, employing the widely used IL17-dependent imiquimod model²² in a direct head-to-head comparison to a high-affinity monoclonal IL17 antibody. As shown in Fig. 4c, serial measurement of ear thickness revealed an approximately 50% reduction of ear swelling in animals receiving either protective vaccination or treatment with IL17A antibody. The same was observed in ex-vivo quantification of epidermal thickness by histology (Fig. 4d/e). Efficacy of the vaccine was also equivalent to the anti-IL17 antibody when tested in dorsal skin (Figure S4). These data suggest that a CMV_{TT} based IL17A-vaccine is equipotent to a monoclonal anti-IL17A antibody in mice.

Immunogenicity under limiting conditions

In clinical practice, eliciting an effective immune response in the vast majority of vaccine recipients can be a major challenge. In psoriasis, an ageing demographic may contribute to a sub-optimal immune responses. In order to obtain evidence whether incorporation of the TT-epitope would boost immunogenicity in poor responders, we emulated sub-optimal vaccine conditions, both by reducing the vaccine dose to the threshold required for a response (0.5 µg) and studying aged mice (10 months). Indeed, as shown in Fig. 4f, we observed an increased formation of IL17A IgG in response to vaccination in mice that had received a DPT-vaccination prior to IL17A vaccination. The same trend was independently observed in young mice (Figure S5). These data suggest that inclusion of a tetanus-derived epitope augments immunogenicity under limiting conditions.

Absence of endogenous IL-17 boosting, reversibility, and ability to re-vaccinate

Finally, we characterized additional parameters important for clinical applications. First, high local IL17A levels in the skin during a psoriasis-flare could theoretically cause an endogenous boosting of anti-IL17A titers. We therefore simulated a psoriasis flare by application of imiquimod cream to mice having received prior anti-IL17A vaccination. However, we did not observe any effect of this treatment on antibody titers (Fig. 4g). Next, we determined long-term titers kinetics. In confirmation of previous experience with VLP-type therapeutic vaccines in humans, anti-IL17A IgG titers were reversible after approximately 10 weeks (Fig. 4h). Importantly, a single 3-monthly vaccine booster shot was able to maintain IL17A IgG titers (white symbols). Furthermore, an alternative boosting regimen applied as single triple-boost after six months was able to re-induce antibody titers without causing high titers 'super boosting'. We conclude that CMV_{TT}-based IL17A vaccination results in potent, reversible, and re-boostable anti IL17A IgG titers in mice, devoid of endogenous boosting during psoriasis-like flares.

Safety of IL17-vaccination

In terms of safety of an IL17-targeting vaccine approach, it is worth noting that, to date, in excess of 5000 patients have been continuously exposed to neutralizing anti-IL17 action (ixekizumab, secukinumab, brodalumab) for at least five years as part of long-term extension clinical trials as well as post-marketing. This extensive clinical dataset has not identified any delayed-onset safety aspects. This duration of IL17 neutralization by far exceeds the expected duration of anti-IL17 suppression effected by a VLP-vaccine based approach, suggesting the latter is unlikely to add safety concerns. In terms of non-target related autoimmune-issues, previous experience with VLP-based vaccines (angiotensin, IL1-targeting vaccines) has not uncovered off-target non-specific T-cell activation facilitating undesired autoimmune effects. Although clinical trials will be required to confirm safety, presently available data therefore do not indicate principle safety limitations of this approach.

Generation of a vaccine against cat allergy

Allergy to cats is highly prevalent and affects up to 10% of the common population. It is not restricted to cat owners,²³ and often has major secondary effects on other atopic conditions such as eczema or asthma. The currently available treatment, specific immunotherapy, lacks consistent efficacy and is fraught with safety issues. Thus, widely available effective treatment for this condition would have significant health benefits. The major allergen responsible for most allergies against cats world-wide is feline domesticus 1 (Fel d 1),²⁴ a 35–39 kDa heterodimer.²⁵ We have previously shown that recombinant Fel d 1 displayed on VLPs derived from the bacteriophage Qβ efficiently confers protection in a murine model of cat allergy.²⁶ To extend these findings to CMV_{TT}-VLPs, recombinant Fel d 1, produced as described previously,²⁶ was coupled to CMV_{TT} via a small Cys containing linker²⁶ (Fig. 5a). To test the immunogenicity of the vaccine, mice were immunized with Fel d 1 either soluble, mixed with CMV_{TT} particles or coupled to CMV_{TT} particles. Fel d 1 coupled to CMV_{TT} was highly immunogenic and induced a 10–100 fold stronger antibody response than free Fel d 1 or Fel d 1 mixed with VLPs (Fig. 5b), demonstrating potent immunogenicity depended on presentation by the CMV_{TT}-VLP.

The main safety limitation of specific immunotherapy against cat allergy is the risk of severe anaphylactic reactions. We therefore tested whether Fel d 1 coupled to CMV_{TT} would carry a lower risk of anaphylaxis than free Fel d 1. To this end, we stimulated basophils in whole blood from an allergic human

donor with either free Fel d 1 or Fel d 1 conjugated to CMV_{TT} and assessed basophil activation. As expected, free Fel d 1 induced strong basophil degranulation over a wide range of concentrations (Fig. 5c). By contrast, degranulation after stimulation with similar amounts of conjugate Fel-CMV_{TT} was greatly reduced (Fig. 5d). Thus, VLP-coupling effectively reduces the anaphylactic reactivity of this potent antigen.

The therapeutic potential of the vaccine Fel-CMV_{TT} was tested next. BALB/c mice were rendered allergic by injection of low doses of natural Fel d 1 in Alum to induce Fel d 1 specific IgE. Mice were then injected either with Fel d 1-CMV_{TT} or unconjugated CMV_{TT} as a control. Two weeks after injection, mice were re-challenged with the allergen and anaphylactic responsiveness was quantified intravital by serial measurement of change in body core temperature

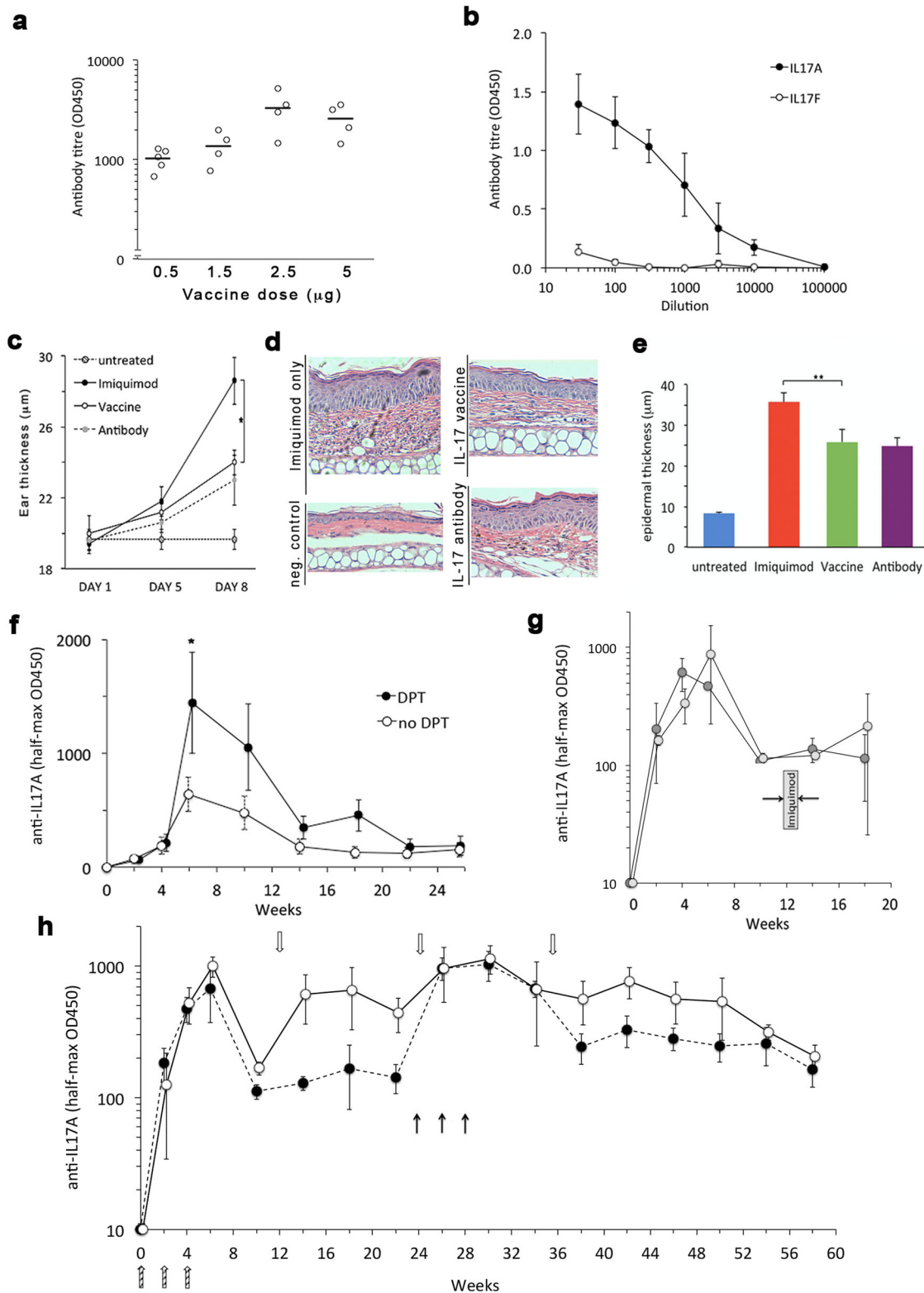


Fig. 4 Immunogenicity and specificity of the immune response generated by vaccination with a CMV-based anti IL17A vaccine in mice. **a** Female C57B/6j mice aged 8 weeks ($n = 4$ per group) were immunized as detailed in the text and anti-IL17A IgG titers determined based on half-maximal OD450 values, as previously described.¹⁸ **b** Specific IgG antibody titers ELISA assays of $n = 4$ mice were performed with plating either recombinant murine IL17A (black circles) or IL17F (open circles). **c** The therapeutic effect of IL17A-vaccination on psoriasis-like disease in mice. Female C57B/6j mice ($n = 5$ per group) were vaccinated three times (day 0, 14, 28) with 50 μ l (2.5 μ g) of vaccine. Two weeks after the last vaccine booster, imiquimod cream was applied daily to the ears and dorsal skin (see Methods). Comparator groups were mice not receiving any treatment, non-immunized mice receiving direct anti-IL17 antibody injection, and untreated mice, as indicated in the figure. Ear thickness was determined on the days indicated. Data shown represent average $\pm$ s.d. * $p < 0.05$ (two-tailed T-test). **d**, representative H&E-based histology of mice shown in **c** sacrificed on day 8. **e**. Quantification of epidermal thickness in all samples. ** $p < 0.01$. **f** The effect of tetanus-pre-vaccination on IL17A-vaccine immunogenicity. Female C57B/6j mice at young (a, 10 weeks) or advanced (b, 10 months) age received either no (open symbols) or DPT-pre-vaccination (closed symbols). Four weeks later, 0.5 μ g of IL17A vaccine was administered on days 0,14,28 and IL17A titers determined by ELISA. * $p < 0.05$ (two-tailed t-test). **g** Endogenous boosting. Female C57B/6j mice ($n = 5$ per group) received triple IL17A vaccination (0,2,4 weeks) followed by no treatment (dark shaded) or one week of imiquimod treatment (light shaded) identical to that employed in Fig. 3. IL17A-IgG titers were measured by ELISA. **h** Long-term anti-IL17A titer kinetics. Female mice were vaccinated at baseline (0,2,4 hatched arrows on bottom), followed by booster vaccination either as single injection every three months (white symbols and arrows), or one single triple injection booster after six months (black symbols). Data shown are average and SEM

(Fig. 5e). Control mice that had received the VLP alone showed a severe drop in temperature, indicative of a strong systemic allergic reaction. In contrast, mice immunized with the vaccine Fel-CMV_{TT} were largely protected (Fig. 5e). Since desensitization to IgE mediated cat allergy ranks as a challenging test for anti-anaphylactic treatments, the data presented here suggest that CMV_{TT} exhibits favorable performance characteristics applicable to the development of therapeutic vaccines for allergies.

Generation of a vaccine against Alzheimer's disease

Alzheimer's disease is one of the biggest issues in global health care. In terms of pathogenesis, treatments targeting both tau-protein (TauRx phase III), as well as β -amyloid²⁷ have yielded disappointing results in clinical trials. Based on recent study results, it is possible that alternative and/or a combination of pathways need to be targeted. In addition, early intervention, preceding clinical symptoms, may be required. In terms of the so-called amyloid hypothesis, there is evidence that early dosing with antibodies specifically targeting N-terminal $\text{A}\beta_{1-42}$ may be effective.²⁸ Clearly, frequent administration of antibodies over decades in patients without clinical symptoms would be impractical. By contrast, a prophylactic vaccination approach could be a viable public health intervention. As most Alzheimer's patients are elderly, the CMV_{TT} platform may confer significant advantages in terms of efficacy. In order to obtain initial data supporting this notion, we tested whether a CMV_{TT} based vaccine against $\text{A}\beta_{1-6}$ would induce the species of antibodies thought to be protective, that is, IgG antibodies recognizing aggregated human $\text{A}\beta_{1-42}$ in the brain of Alzheimer's patients. To this end, we coupled $\text{A}\beta_{1-6}$, i.e., the N-terminus of $\text{A}\beta_{1-42}$ to CMV_{TT} ($\text{A}\beta_{1-6}$ -CMV_{TT}) or VLPs without the tetanus epitope ($\text{A}\beta_{1-6}$ -CMV_{WT}, Fig. 6a). The control vaccine as such is analogous to the CAD106 vaccine currently undergoing clinical testing (NCT02565511), as both are VLP-based vaccines coupled to the same epitope.

In order to show enhanced immunogenicity by vaccination with Abeta-CMV containing the TT epitope, 8 weeks old C57BL/6 mice were primed with Infanrix, a hexa-valent vaccine for children against Tetanus and other infections e.g., diphtheria, pertussis, hepatitis B, polio, and haemophilus influenza B. After four weeks, mice either primed with Infanrix or left untreated were immunized with 10 μ g, 1 μ g or 0.1 μ g of Abeta-CMV TT vaccine and the anti-Abeta IgG response in serum was analyzed (Fig. 6b). In comparison to the unprimed control group, mice that were primed with Infanrix showed an increased immune response especially early on day 5 and 7 after vaccination against Abeta peptide independent of the dose amount.

In order to study whether the increased immunogenicity also holds true in older animals, six months old BALB/c mice were either pre-immunized against Tetanus or left untreated as control before immunization with 1 μ g of $\text{A}\beta_{1-6}$ -CMV_{TT} or $\text{A}\beta_{1-6}$ -CMV_{WT}.

Vaccination with CMV_{TT} compared to CMV_{WT} vaccine already induced a stronger response regardless of whether mice had been primed against tetanus (Fig. 6c, compare "only $\text{A}\beta$ -CMV_{TT}" with "Infanrix + $\text{A}\beta$ -CMV_{WT}"). Immunogenicity of $\text{A}\beta$ -CMV_{TT} vaccine was even further increased when mice were primed against tetanus (Fig. 6c, compare "Infanrix + $\text{A}\beta$ -CMV_{TT}" with "only $\text{A}\beta$ -CMV_{TT}"). Thus, young and old mice immune to tetanus mount an enhanced response to an Alzheimer's vaccine based on CMV_{TT}.

To assess whether the induced antibodies exhibited the right specificity, serum antibodies of mice immunized with $\text{A}\beta_{1-6}$ -CMV_{TT} vaccine were tested for their ability to recognize plaques of $\text{A}\beta$ in brain sections from Alzheimer's patients (Fig. 6d-f). Antibodies induced by active immunization with the $\text{A}\beta_{1-6}$ -CMV_{TT} vaccine (Fig. 6f) recognized Alzheimer's plaques on human brain sections similar to as a monoclonal antibody raised against $\text{A}\beta$ peptide 1-17 (Fig. 6d). Serum from immunized but not control mice recognized both $\text{A}\beta_{1-42}$ by ELISA (not shown) as well as plaques in brain sections from Alzheimer's patients, indicating that $\text{A}\beta_{1-6}$ -CMV_{TT} was able to induce antibody responses of the desired specificity (Fig. 6e,f).

METHODS

Ethics statement

All studies involving either animal and/or human samples were subject to prior approval by the respective local ethics committees. For studies using mice, methods were performed in accordance with relevant regulations and guidelines. Methods were approved by the animal ethics research committee of the University of Dundee as part of the standard operating procedure for study approval for project licence 60/4944. Studies involving human tissue for Alzheimer histology were overseen by KEK Zürich ethics committee. Tissue was ascertained as part of a KEK-approved project (Adriano-Aguzzi/i2016). According to local governance regulation, use of anonymised slides for KEK-approved projects involving post mortem tissue is not subject to post-hoc individual consenting.

Isolation and cloning of a coat protein (CP) of cucumber mosaic virus (CMV)

The total RNA from CMV-infected lily leaves collected from a private garden in Riga, Latvia, was isolated using TRI reagent (Sigma, Saint Louis, USA) in accordance with manufacturer's recommendations. For cDNA synthesis, OneStep RT-PCR kit (Qiagen, Venlo, Netherlands) was used. For amplification of the CMV CP gene, the primer sequences were chosen following analysis of CMV sequences from GenBank: CMcPF (CACCATG-GACAAATCTGAA TCAACCACTGCTGGT) and CMcPR (CAAAGCTTAT-CAAACTGGGAGCA CCCAGATGTGGGA); NcoI and HindIII sites underlined. The corresponding PCR products were cloned into the pTZ57R/T vector (Fermentas, Vilnius, Lithuania). *E. coli* XL1-Blue cells were used as a host for cloning and plasmid amplification. To avoid RT-PCR errors, several CP gene-containing pTZ57 plasmid clones were sequenced using a BigDye cycle sequencing kit and an ABI Prism 3100 Genetic analyzer (Applied Biosystems, Carlsbad, USA). After sequencing, the cDNA

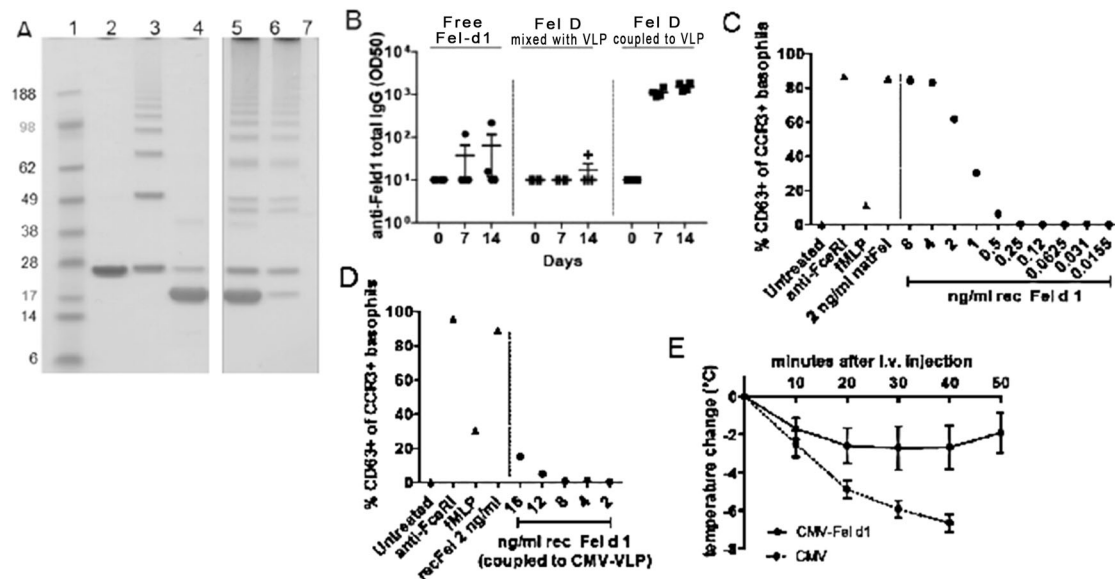


Fig. 5 Generation of a vaccine against cat allergy. **a** Analysis of the recombinant Fel-CMV_{TT} vaccine. SDS Page (NuPage 4–12% Bis-Tris) was run under denaturing and reducing conditions. Lanes from left to right: 1-MW markers (Invitrogen, SeeBlue Prestained), 2-purified CMV VLP (~25 kDa coat protein monomer), 3-derivatized CMV_{TT}-VLP after reaction with SMPH (bands in order of ascending size corresponding to VLP monomers, dimers, trimers etc. resulting from cross-linking), 4-purified Fel d1 (~20 kDa), 6/7-vaccine Fel-CMV_{TT} following conjugation with Fel d1 protein, loaded amount of VLP 5 µg and 10 µg, 9/10-Fel-CMV_{TT} after removal of uncoupled Fel d1 by ultrafiltration, loaded total protein amount of VLP 5 µg and 10 µg. Samples were derived from the same experiment and were processed in parallel. **b** Immunogenicity assessment in Balb/C mice that received 1 µg of Fel d1 protein in three versions: free, mixed with or coupled to CMV_{TT} intravenously on day 0. Blood was collected on days 0, 7 and 14 and analyzed for Fel d1 specific total IgG titers (OD50). **c, d** Basophil activation test was performed with whole blood from a cat-allergic patient. Blood samples were incubated with different amounts of free recombinant Fel d1 **c** or recombinant Fel d1 coupled to CMV_{TT} **d**. Untreated, anti-FceRI, fMLP and natural Fel d1 served as controls. **e** Fel d1 allergen challenge *in-vivo*. Mice were sensitized with natural Fel d1 and vaccinated with Fel-CMV_{TT} vaccine or CMV_{TT} VLP alone. To show protection of sensitized mice upon immunization, mice were challenged with 3 µg natural Fel d1 in 150 µl PBS i.v. Mean temperature changes (°C) in Fel-CMV_{TT} vaccine or CMV_{TT}-VLPs-vaccinated mice +/- SEM are shown (n = 5). After 40 min CMV_{TT}-vaccinated mice were sacrificed because of severe anaphylactic symptoms

of CMV CP gene without sequence errors coding for CMV coat protein was then subcloned into the NcoI/HindIII sites of the pET28a(+) expression vector (Novagen, San Diego, USA), resulting in the expression plasmid pET-CMV_{WT}.

To replace the original amino acids at the N-terminus of CMV CP against foreign epitope sequences, as a template for PCR amplification and mutagenesis the pET-CMV_{WT} plasmid was used. Internally in CMV_{WT} gene located SalI site (Fig. 1) was used for cloning corresponding PCR products.

To introduce the tetanus toxoid epitope coding sequence in CMV_{WT} gene, two step PCR mutagenesis was necessary. For the first step amplification following primers were used for PCR: pET-220 (agcaccgc cgccgaaggaa –upstream from pET28a+ polylinker, the amplified region includes BglII site) and CMV-tt83-1R (ATTGGAGTTGGCCTTAATATACT GGCCATGATATATCTCTTCTTAAAGT). For the second round the PCR product from the first round was diluted 1:50 and re-amplified with primers pET-220 (SEQ ID NO: 11) and CMV-tt83Sal-R2 (GACGTC-GACGCTCGGTAATCCCGATAAATTGGAGTTG GCCTTAATATACTG).

To obtain CMV VLPs, *E. coli* C2566 cells (New England Biolabs, Ipswich, USA) were transformed with the CMV_{TT} CP gene-containing plasmid pET-CMV_{TT}. After selection of clones with the highest expression levels of target protein, *E. coli* cultures were grown in 2xTY medium containing kanamycin (25 mg/l) on a rotary shaker (200 rev/min; Infors, Bottmingen, Switzerland) at 30 °C to an OD₆₀₀ of 0.8–1.0. Then, the expression was induced with 0.2 mM Isopropyl-β-D-thiogalactopyranoside (IPTG), and the medium was supplemented with 5 mM MgCl₂. Incubation was continued on the rotary shaker at 20 °C for 18 h. 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, 5 mM mercapto-ethanol (pH 9.0, buffer A) and were disrupted by ultrasonic treatment. Insoluble proteins and cell debris were removed by centrifugation (13,000 rpm, 30 min at 5 °C). The soluble CMV_{TT} CP protein in clarified lysate was pelleted using saturated ammonium sulfate (1:1, vol/vol) overnight at +4 °C. Soluble CMV_{TT} CP-containing protein solution was separated from the cellular proteins by ultracentrifugation (SW28 rotor, Beckman, Palo Alto, USA; at 25,000 rpm, 6 h, 5 °C)

in a sucrose gradient (20–60% sucrose in buffer A, without mercapto-ethanol, supplemented with 0.5% Triton X-100). After dialysis of CMV-containing gradient fractions, VLPs were concentrated using ultracentrifuge (TLA100.3 rotor, Beckman, Palo Alto, US; at 72,000 rpm 1 h, +5 °C) or by ultrafiltration using Amicon Ultra 15 (100 kDa; Merck Millipore, Cork, Ireland). All steps involved in the expression and purification of VLP were monitored by SDS-PAGE using 12.5% gels.

Characterization of CMV_{TT} VLPs

The concentration of purified CMV_{TT} was estimated using the QuBit fluorometer in accordance with manufacturer's recommendations (Invitrogen, Eugene, USA). Concentrated VLP solutions (approximately 3 mg/ml) were stored at +4 °C in 5 mM sodium borate, 2 mM EDTA, buffer (pH 9.0). To demonstrate the presence of tetanus epitope in CMV VLPs, the mass spectrometric analysis of the purified CMV_{TT} (also referred to as CMV-Ntt830) VLPs was used. The stability of VLPs was investigated by thermal denaturation in the presence of SYPRO-orange dye using a DNA melting point determination program and a real-time PCR system MJ Mini (Bio-Rad, Hercules, USA). This involved subjecting VLP samples to increasing heat whilst monitoring fluorescence. The morphology of VLPs was confirmed by applying samples (0.1–0.5 mg/mL) on glow discharged carbon coated copper grids and observing by transmission electron microscopy using JEM-1230 electron microscope (JEOL, Tokyo, Japan). Average sizes of VLP preparations were determined by dynamic light scattering, assessed by diluting to 1 mg/ml and analyzing in Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Malvern, UK).

Trypsin-fingerprint/mass spectrometry

Reactive Lys residues available for chemical coupling of antigens were determined by FITC-labeling free amino groups, FITC-modified coat protein was separated in SDS/PAGE gel, the fragment was excised from gel and digested with trypsin (Trypsin Profile IGD Kit, Sigma). VLPs (1.5 mg/ml in 50 mM Sodium borate, 2 mM EDTA, pH 9.0) were reacted with 20 mM FITC at

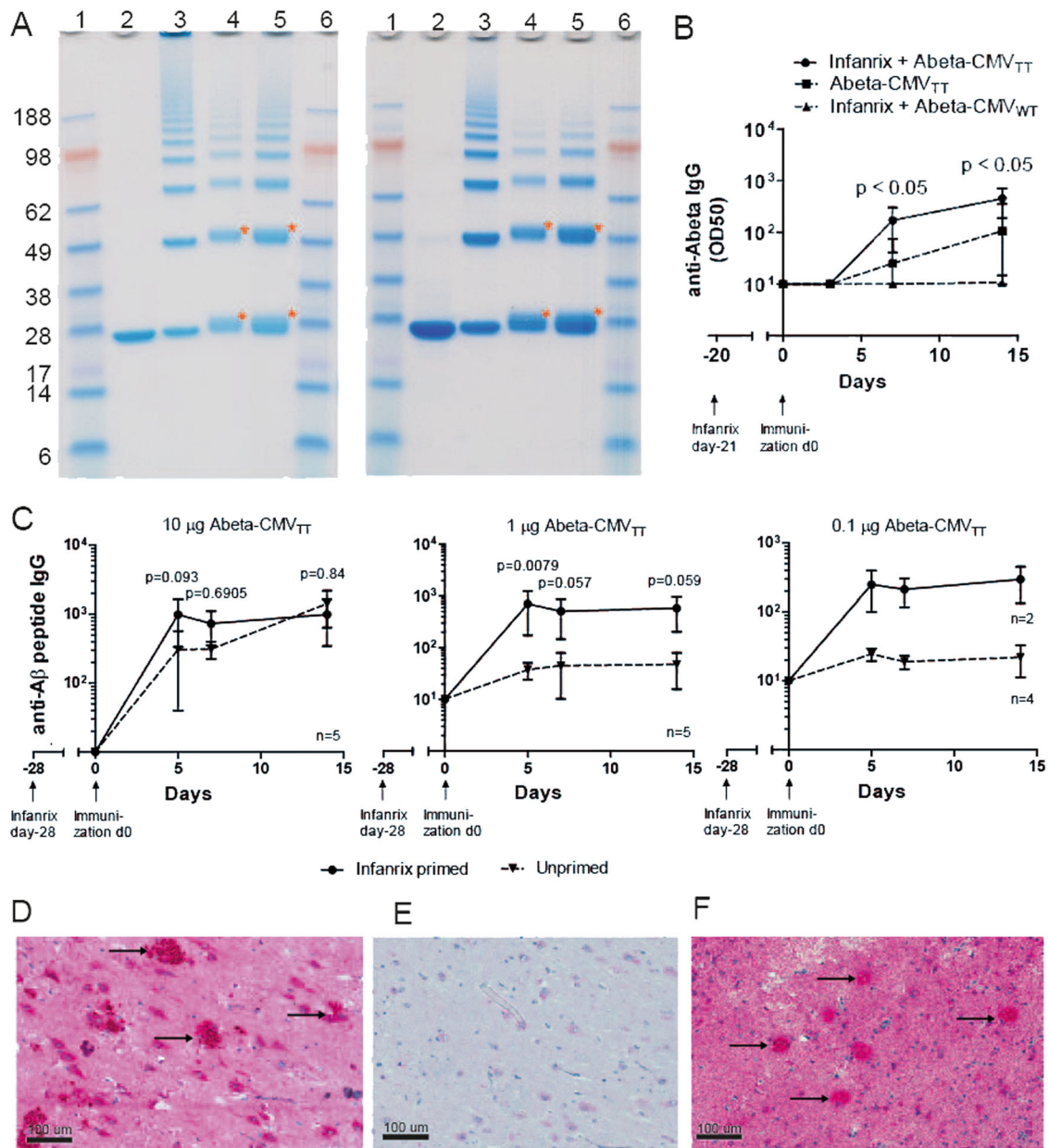


Fig. 6 Immunogenicity of CMV_{TT}-based Alzheimer's vaccine. **a** SDS-PAGE analysis (NuPage 4–12% Bis-Tris) of the Aβ₁₋₆-CMV_{TT} (left panel) and Aβ₁₋₆-CMV WT (right panel) vaccine was run under denaturing and reducing conditions. Lanes from left to right: 1/6 - MW markers (Invitrogen, SeeBlue Prestained), 2-purified CMV VLP (~25 kDa coat protein monomer), 3-derivatized CMV-VLP after reaction with SMPH (bands in order of ascending size corresponding to VLP monomers, dimers, trimers etc. resulting from cross-linking), 4/5-Aβ-CMV following conjugation of the antigen amount loaded 5 μg (4) or 10 μg (5). Samples were derived from the same coupling experiment and were processed in parallel. **b** Immunogenicity assessment in young mice. One group of eight weeks old female C57BL/6 mice were primed with Infanrix and the other group was left untreated. After four weeks, mice were immunized with 10 μg, 1 μg or 0.1 μg of Aβ₁₋₆-CMV_{TT} vaccine. Blood was collected on day 0, 5, 7 and 14 and analyzed for Aβ-specific total IgG titers (endpoint). Mean values with SEM are shown of a representative experiment with *n* = 2–5 as indicated. Significances were obtained by a Mann Whitney test. **c** Immunogenicity assessment in old mice. Six months old Balb/C mice were primed with Infanrix® or left untreated. After 3 weeks, mice received 1 μg of Aβ₁₋₆-CMV_{TT} or 1 μg of Aβ₁₋₆-CMV WT vaccine intravenously. Blood was collected on day 0, 3, 7 and 14 and analyzed for Aβ-specific total IgG titers (OD50). Mean values with SEM are shown of a representative experiment with *n* = 8. Significances with *p* < 0.05 were obtained by a Mann Whitney test. **d–f** Immunohistochemistry of human brain section of the hippocampus showing the dentate gyrus with Cornu Ammonis of an Alzheimer's patient. Formalin-fixed in paraffin embedded brain tissue of an Alzheimer's patient was stained with **d** a mouse mAb generated against the Aβ peptide 1–17 as a positive control, **e** naïve mouse serum from day 0 as negative control and **f** purified serum from mice immunized with Aβ-peptide 1–6 (from N-terminus) coupled to CMV_{TT} VLP from day 56. All samples were counterstained with hematoxylin. Dimensions are depicted in pictures (100 μm). Arrows indicate single Aβ plaques.

+4 °C for 24 h. Samples after FITC treatment were separated in 12% SDS-PAGE, protein spots after Coomassie staining excised from gel and treated with Trypsin using Trypsin Profile IGD Kit and protocol (Sigma, St Louis, USA) ON +4 °C. The reaction mixture was purified using ZipTip tips (Merck

Millipore, Cork, Ireland), diluted with a 3-hydroxypicolinic acid matrix solution and spotted onto an MTP AnchorChip 400/384TF. MALDI-TOF MS analysis was carried out on an Autoflex MS (Bruker Daltonik, Bremen, Germany). The protein molecular mass (MM) calibration standard I (3–20

kDa; Bruker Daltonik) and Peptide calibration standard II was used for mass determination.

T cell proliferation assays

Blood was obtained from human volunteers and peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll Paque (GE Healthcare, Chalfont St. Giles, UK). PBMCs were labeled with 10 μ M CFSE (Biolegend, San Diego, CA, USA) according to the manufacturer's protocol and cultured in RPMI-1640 (Gibco, Basel, Switzerland) supplemented with 5% of heat-inactivated human AB serum (Swiss Red Cross, Bern, Switzerland), 2 mM L-Glutamine (Biochrom, Berlin, Germany), 50 U/ml penicillin and 50 μ g/ml streptomycin (Bioconcept, Allschwil, Switzerland). During 7 days, the cells were stimulated with either 1 μ g/ml CMV WT or CMVTT or left untreated. T cells were then stained with anti-CD3-PerCp-Cy5.5, anti-CD4-PE-Cy7 (Biolegend, San Diego, CA, USA), data acquired with BD FACSCanto™ and analyzed with FACS-Diva software (BD Biosciences, Franklin Lakes, NJ, USA). This study was approved by the local ethics committee.

CryoEM reconstruction to determine CMV_{TT} VLP structure

CMV_{TT} (2.5 mg/ mL) in 5 mM sodium borate, 2 mM EDTA, pH 9 was applied for 20 s to glow-discharged holey carbon-coated copper grids (C-flat, CF-2/1-2 C; Protochips) in a humidified chamber (70% relative humidity), excess sample being removed by blotting on to filter paper and grids rapidly vitrified by plunging in to liquid ethane (Vitrobot, FEI). Cryo-grids were stored in liquid nitrogen prior to imaging with a Tecnai F30 Polara microscope (FEI).

CryoEM images were collected using a Tecnai F30 'Polara' microscope (FEI) at 300 kV, equipped with an energy filter (GIF Quantum, Gatan) operating in zero-loss mode (0–20 eV energy selecting slit) and a direct electron detector (K2 Summit, Gatan). Movies (25 frames, each 0.2 s) were recorded at 1.0–3.0 μ m underfocus in single-electron counting mode with SerialEM at a calibrated magnification of 37,037 $\times$, thus resulting in a pixel size of 1.35 Å. Frames from each movie were aligned and averaged to produce drift-corrected micrographs.²⁷ Data are summarized in Table S1.

Structures were solved with RELION 1.3 according to recommended gold-standard refinement procedures,²⁸ and icosahedral symmetry was applied. Micrographs showing signs of astigmatism or significant drift were discarded and not used for further analysis. Reference-free 2D class averaging was used to discard distorted particles. The particle population was further improved by 3D classification. The X-ray structure of native CMV (PDB: 1F15)²⁹ was low-pass-filtered to 50 Å and used as an initial template for 3D classification and refinement. A total of 3582 CMV_{TT}830 particles from 6600 micrographs were used to solve the final density maps at 4.2 Å resolution, as indicated by Fourier shell correlation and 0.143 cut-off.

Using a similar approach as previously described,^{30,31} the CMV was fitted in the density map as a rigid body with UCSF Chimera 3.2. The fitting was improved further by real-space refinement using COOT 3.3 and Phenix 3.5. Only the coordinates being refined each time, with the maps kept constant. Cross-correlation guided each round of model optimization between the map and the model.

Production of mIL17 protein

A nucleotide fragment codon optimized for *E. coli* was synthesized (GeneArt Genestring, ThermoFisher Scientific, UK) encoding the murine IL-17 (aa 26–158, accession: NP_034682) preceded by sequences for an initiating methionine and glycine and coding region for a hexahistidine tag at the N-terminus of the encoded protein, was followed by additional sequences to give a polylinker of 5 glycine and terminal cysteine at the C-terminus. This fragment was cloned by restriction digest (NcoI/ BamHI, NEB, UK) in to a similarly double digested prokaryotic expression vector (pET28b, Merck, UK). Resulting clones were confirmed by PCR colony screen and sequenced using T7 promoter forward primer (TAATACGACTCACTATAGGG) and pET-RP reverse primer (CTAGTTATTGCTCAGCGG).

Murine IL-17 was expressed in *E. coli* strain BL21(DE3)Star (Thermo Fisher Scientific, UK), culturing to OD₆₀₀ of 0.7 and inducing with 1 mM IPTG overnight (16–18 h) at 37 °C. Harvested cells were lysed in PBS, pH 7.4 supplemented with Lysozyme™ as per manufacturers recommendation (Merck, UK), incubating 1 h and sonicating lysate on ice (3 cycles of 20 s on, 20 s off, 40% power amplitude with a VibraCell fitted with microtip, Sonics and Materials Inc, US). The lysate was clarified by high speed centrifugation (15,000 $\times$ g, 30 min) and the pellet was retained. The recombinant IL-17 was

purified from the insoluble fraction as previously described.¹⁸ Briefly, inclusion bodies (IBs) from the insoluble fraction were subjected to sequential washes with 50 mM Tris pH 8, 10 mM EDTA, 100 mM NaCl buffer (initially containing 0.5% Triton $\times$ 100 then 2% Triton $\times$ 100, then without detergent). The IBs were solubilized with solubilization buffer (6 M guanidine HCl, 10 mM Tris.HCl, 100 mM NaH₂PO₄, 2 mM beta-mercaptoethanol (BME), pH 8), insoluble debris removed by centrifugation at 10,000 g, 20 min and clarified soluble fraction subjected to nickel affinity chromatography (HisTrap Excel, GE Healthcare) under denaturing conditions with low pH elution (50 mM NaH₂PO₄, 100 mM Tris, 6 M Gdn.HCl, 2 mM BME with Buffer A, adjusted to pH 6.15; and Buffer B adjusted to pH 4). This was followed by concentration (Amicon Ultra 3000 centrifugal filters, Merck, UK) and further purification using size exclusion chromatography (HiLoad Sephadex 75, GE Healthcare, UK).

Fractions containing purified mIL-17 as identified by a 16 kDa band (predicted molecular mass of 16,378 Da) by Coomassie stained SDS-PAGE were pooled. Note that proteins were ethanol precipitated and resuspended in reducing Laemmli buffer to remove guanidine prior to the analysis by SDS-gel electrophoresis. The purified mIL-17 was still denatured and therefore required refolding to native form. Sample was adjusted to a concentration of <0.2 mg/mL and refolding followed a 2-step dialysis strategy. Dialysis tubing was subject to 3 buffer exchanges (2 L each) over 24 h with 0.5 M Arginine, 50 mM sodium phosphate (pH 8), 10% (v/v) glycerol and redox pair (5 mM reduced glutathione, 0.5 mM oxidized glutathione). For the second step of dialysis, samples were dialyzed for a further 24 h in to 50 mM sodium phosphate (pH 8), 10% (v/v) glycerol, with at least 2 buffer exchanges (2 L each).

Production of psoriasis vaccine

The purified and refolded mIL-17 was conjugated to CMV_{TT} via the heterobifunctional crosslinker, Succinimidyl-6-[(beta-maleimidopropionamido)hexanoate] (SMPH, Thermo Fisher Scientific, UK). VLPs were reacted with 7.5-molar excess of SMPH, for 30 min at room temperature (RT), unreacted excess crosslinker being removed by 3 repeated diafiltration steps using 100 k centrifugal filters. The recovered derivatised VLPs were then mixed at equimolar concentration with mIL17 that was pre-treated 60 min at RT with 10-molar excess TCEP to liberate C-terminal cysteines (dimeric IL-17 has intramolecular disulfide bonds forming a cysteine knot that are typically resistant to reducing reagents without denaturation). Coupling efficiency was estimated by densitometry analysis of CMV_{TT} bands in Coomassie stained SDS-PAGE, comparing proportions of monomer net intensity (NI) and monomer plus IL-17 NI after correcting for relative MW (See figure S3B). Densitometric values were provided using Image Lab 5.0 software (Bio-rad Laboratories) or equivalent image analysis software. Coupling or epitope density for IL17 per VLP was calculated as follows (eq1): IL17-CMV(NI/MW) / (Σ VLP(NI/MW) + IL17-CMV(NI/MW)) $\times$ number of protein per subunit = epitope density (ED). Expressed as a percentage this gives an estimated coupling density of IL17-CMV of 8%, meaning a minimum of 14 molecules of IL17 were covalently linked to each particle (consisting of 180 CMV monomers).

In vivo clinical efficacy and immunogenicity of the IL17 vaccine in mice

In vivo clinical efficacy was measured by dorsal skin and ear epidermal thickness assessed macroscopically and with H&E histology. Ear thickness measurements and body weight measurements were recorded at different time intervals during the experiments using a micrometer (Mituyo catalog nr 7301, Thickness Gage Series 7 Flat Anvil Type Dial) and a scale, respectively. Ear samples and dorsal skin samples were taken, roughly 2 cm by 2 cm, and fixed in neutral buffered 10% formalin. 2 ml of formalin was used per 100 mg of tissue. Tissues were fixed for a minimum of 48 h at RT. Tissue was then dehydrated through a series of graded ethanol immersions. Once fixed, the tissue was processed as follows on a Leica Peloris tissue processor: formalin $\times$ 2, 95% ethanol $\times$ 4, 99% ethanol $\times$ 4, xylene, 99% ethanol first paraffin wax and second paraffin wax. Wax was melted by placing tissue cassettes in 58 °C paraffin bath for 15 min. Mold that left 2 mm margin of paraffin wax around the tissue was selected. Molten paraffin was dispensed in mold and warm forceps were used to transfer tissue into the mold, placing the cut side down. The mold was transferred to a cold plate and gently the tissue was pressed flat. With the tissue in the desired orientation the labeled tissue cassette was added on top of the mold as a backing. After 30 min the wax was cooled and hardened and the paraffin block is popped out of the mold. Tissues were

then sectioned using a Leica microtome to 4 μ m thickness. The sections were dried in an oven at 37 °C. The tissue and paraffin attached to the cassette has formed a block, which was stored at RT. Haematoxylin and Eosin staining was performed using Leica Autostainer XL. The procedure was as follows: tissue was placed in an oven at 60 °C for 15 min. Deparaffinise sections, 3 changes of xylene, 30 s each. Re-hydrate in 2 changes of absolute alcohol, 30 s each. 99% alcohol for 2 min and 95% alcohol for 2 min. Wash in water for 30 s. Stain in Harris haematoxylin solution for 4 min. Wash in running tap water for 1 min. Differentiate in 0.1% acid alcohol for 1 min. Wash in water for 1 min. Bluing in saturated lithium carbonate solution for 1 min. Counterstain in eosin solution for 20 s. Wash in tap water for 30 s. Rinse in 95% alcohol for 30 s $\times$ 2 and rinse in 99% alcohol for 30 s $\times$ 2. Rinse in Isopropyl alcohol for 30 s. Clear in 3 changes of xylene, 30 s each. Sections were coverslipped by Leica Coverslipper CV5030 using DPX mountant.

Immunogenicity was assessed via measuring anti-IL-17A serum levels with a series of ELISA experiments. Serum samples were acquired at baseline, at each vaccination point and 2 weeks post final vaccination. Samples of up to 200 μ l were acquired at each point with tail vein blood sampling. First Nunc-Immuno 96 clear polystyrene MicroWell solid plates were coated as follows. Recombinant murine (rm) IL-17 was diluted to a concentration of 2 μ g/ml in PBS and vortexed to allow adequate mixing. 100 μ l of this mixture was added to each well of the plate using a multi-channel pipette. The plate was covered with parafilm and stored at 4 °C for up to 72 h. Wash buffer was made using PBS with 0.05% Tween20 and blocking buffer made from the washing buffer composed of 2% BSA in PBS with 0.05% Tween20. The plate was removed from the fridge, the liquid expelled and the plates washed 3 times with 200 μ l wash buffer per well. 250 μ l blocking buffer was added to each well and the plates left for 150 min. Serum was serially diluted in wash buffer on 96-well plates to the concentrations 1:10, 1:100 etc. Control serum, taken before any antibody or vaccination injections, was used in each experiment and diluted to a concentration of 1:100. This high concentration relative to the test samples was used to allow for enough serum to be used for all the experiments. Liquid was discarded from the blocked plate and the plate was washed 3 times as above. 50 μ l of diluted serum was transferred onto the coated plate, with each sample a duplicate of that sample was used. Empty wells were filled with 50 μ l sterile water for standardization purposes and the plate incubated for 90 min on the shaker. Detection antibody was made using Anti-mouse IgG labeled to alkaline phosphatase diluted in blocking buffer to a concentration of 1:5000. Liquid was discarded from the blocked plate and the plate was washed 3 times as above. 100 μ l of detection antibody was added to each well and the plates covered with parafilm and left to incubate on the shaker for 45 min. Liquid was discarded from the blocked plate and the plate was washed 3 times as above. 50 μ l of alkaline phosphatase yellow (pNPP) liquid substrate was added to each well and the wells incubated on the shaker for 20 min. 5 μ l of stop solution (3 M NaOH) was added to each well and the plate read in the SpectraMaxM3 spectrophotometer at 405 nm. SoftMax Pro6.3 software was used to read the plates. Absorbance instruments with endpoint settings of 405 nm wavelength were used. ELISA data were analyzed using a 3-parameter logistic fit according to standard sigmoid curve equation, $Y = \text{MAX}/(1 + (x/\text{EC}_{50})^{\text{SLOPE}})$ where the minimum was set to zero after subtracting the H₂O optical densities from test values. Curve fitting was done using the 'solver' add-in using the GRC-nonlinear fitting algorithm.

Residual tail vein blood samples known to be highly immunogenic for IL-17A were used to test the specificity of the immune response generated by the vaccine on ELISA plates coated with IL-17A and IL-17F. The same blood samples were tested for both IL-17A and IL-17F and the ELISA was run for both at the same time to minimize variables.

Expression and purification of Fel d 1

The sequence of Fel d 1 antigen^{25,29,30} comprises of chain 1 and chain 2 that were genetically fused via a 15-aa linker sequence (GGGGS)₃ and synthesized by GeneArt (Thermo Fisher Scientific, Germany). The COOH-terminus was modified by a histidine tag (6x His) for purification purposes followed by a small spacer (GGCG) including a cysteine for coupling. Our version of the recombinant Fel d 1 has a predicted molecular mass of 20,147 Da. The encoding Fel d 1 sequence was cloned into the high level-expression plasmid vector pET42. *E. coli* C25661 (NEB) were transformed with the plasmid and cultivated at 37 °C. At a OD₆₀₀ between 0.5 and 0.7 1 mM IPTG was added to the culture and placed at 20 °C overnight for soluble protein expression. Cells were harvested and separated from supernatant by centrifugation at 4800 $\times$ g for 15 min at 4 °C. The cell pellet

was resuspended in 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0 and sonicated for cell lysis. The solution containing the recombinant Fel d 1 was cleared from cell debris by centrifugation at 16,000 $\times$ g for 30 min at 4 °C. The supernatant was collected and purified by a Ni-NTA (Qiagen) column using the ÄKTA purifier. Recombinant Fel d1 containing a His-tag bound to Ni-NTA and was washed with 50 mM NaH₂PO₄, 300 mM NaCl, 50 mM imidazole, pH 8.0, finally eluted in 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0 and dialyzed against PBS overnight at 4 °C. The antigen authenticity was tested in a capture ELISA (EL-FD1, Indoor Biotechnology) using mAbs that were raised against natural Fel d 1.

Production and immunogenicity testing of Fel d 1 vaccine

The purified Fel d 1 was conjugated to CMV_{TT} VLPs using the crosslinker SMPH. A 10-molar excess of SMPH reacted at 23 °C for 60 min shaking at 350 rpm. The side product was removed using PD10 desalting columns (GE Healthcare). To gain access to the cysteine at the COOH-terminus of the recombinant Fel d 1, the antigen solution reacted with a 10-molar excess of a mild reducing agent TCEP (Sigma) before a 2-molar excess of Fel d 1 was added to the derivatized CMV_{TT}-VLP at 23 °C for 3 h at 350 rpm. Unbound Fel d 1 protein was removed by Amicon Ultra centrifugal filter devices (Millipore) and tested by SDS-PAGE analysis. CMV monomers appear at ~25 kDa and Fel d 1 protein at ~20 kDa. Due to crosslinking of subunits, derivatization by SMPH leads to the characteristic ladder of CMV monomers, dimer, trimers, tetramers, etc. The primary coupling band appears as one CMV monomer linked to one Fel d 1 protein at ~45 kDa. Coupling efficiency was calculated by densitometry (as previously described for IL17A-CMV_{TT} vaccine) with a result of ~30% meaning 60 Fel d 1 molecules were linked to one particle.

To test immunogenicity, 8 weeks old Balb/c were immunized with 1 μ g of free Fel d 1, Fel d 1 mixed with or coupled to CMV_{TT} VLPs in 150 μ l PBS intravenously. Blood was collected at different time points and analyzed for anti-Fel d1 antibodies by ELISA. Natural Fel d 1 (Indoor Biotechnologies) at 1 μ g/ml was applied overnight to NUNC ELISA plates which were then washed and blocked with 2% BSA in PBS Tween 20 (0.05%). After washing, serially diluted mouse sera were applied to the plates. After further washing, goat anti-mouse IgG antibody labeled with horseradish peroxidase was applied to the plates. Following a final washing step, O-phenylenediamine dihydrochloride was added and, after 7 min, the reaction stopped with 5% sulfuric acid. The conversion of OPD by HRP was measured at 450 nm. The titers is reported as OD₅₀ which is the reciprocal of the dilution which reaches half of the maximal OD value.

Basophil activation test (BAT)

The BAT assay (Bühlmann Co.) using whole blood from allergic patients was tested for the up-regulation of the degranulation marker CD63 on basophils (CCR3+)³¹ upon incubation with Fel d 1 and Fel d 1 couples to CMV_{TT}-VLPs by flow cytometry using a BD Fortessa. Briefly, 100 μ l of stimulation buffer was mixed with 50 μ l of EDTA-treated whole blood. In addition, 50 μ l of dilutions of natural Fel d1, recombinant Fel d1 and recombinant Fel d 1 conjugate (Fel-CMV_{TT}) were added. Positive control solutions including an mAb against Fc ϵ R1 as well as an unspecific cell activator (fMLP) were also tested in the assay. Staining dye (20 μ l per sample), containing anti-CCR3 Ab labeled to PE and anti-CD63 Ab labeled to FITC, was added and incubated at 37 °C for 25 min. Erythrocytes were subsequently lysed adding lysis buffer. After 10 min incubation, the samples were centrifuged at 500 $\times$ g for 5 min and washed with wash buffer (PBS containing 2% FCS). After a second centrifugation step, the cell pellets were resuspended in wash buffer and acquired using a flow cytometer (FACS BD Fortessa). The samples were analyzed by DIVA software. The percentage of the CD63 expression on CCR3+ basophils was analyzed.

Fel d 1 allergen challenge *in-vivo*

For Fel d 1 sensitization, two groups of 5 female 6-wk-old BALB/c mice were injected with 1 μ g natural Fel d 1 (Indoor biotechnologies) mixed in 200 μ l Alum (10 mg/ml Al(OH)₃; Alhydrogel adjuvant 2%, InvivoGen) at day 0, 7 and 14 intraperitoneal. At day 28 mice were vaccinated with 30 μ g vaccine Fel-CMV_{TT} or only CMV_{TT}-VLPs subcutaneously.

For the induction of anaphylaxis, two weeks after vaccination on study day 42 mice were challenged with 3 μ g natural Fel d1 in 150 μ l PBS intravenously. The rectal core temperature was measured with a rectal probe digital thermometer (MiniTemp, Vetricon Services LTD) every 10 min for 50 min after intravenous antigen challenge.

Production of Alzheimer's vaccine

CMV_{TT} VLPs or CMV wildtype (WT) VLPs reacted with a 7.5-molar excess of the crosslinker SMPH for 1 h at RT. A short 6-aa sequence (DAEFRH) from the N-terminus from the full length A β peptide (1–42 aa) encoding with terminal cysteine residue (GGC) purchased from Dr. Petra Henklein, Charite, Berlin, Germany was conjugated in a 5-molar excess to the derivatised CMV_{TT} or CMV_{WT} particles for 3 h at RT. The vaccine was analyzed by SDS-PAGE to confirm coupling of peptide to CMV_{TT} or CMV_{WT} particles. CMV monomers appear at ~25 kDa. Due to crosslinking of subunits, derivatization by SMPH leads to the characteristic ladder of CMV monomers, dimer, trimers, tetramers, etc. Coupling bands appear above CMV monomer when one peptide (~1 kDa) bound to CMV monomer (coupling product ~26 kDa) or two peptides bound to CMV monomer (coupling product ~27 kDa). Coupling bands of peptide(s) to CMV dimers appear above CMV dimer accordingly. The coupling efficiency (described previously for IL17A-CMV_{TT} vaccine) was calculated by densitometry resulting in ~60% meaning approximately 108 peptide molecules were linked to each VLP.

Immunogenicity assessment of Alzheimer's vaccine and assessment of TT enhancement

To test the TT enhancement, 6 months old female Balb/C mice were primed with Infanrix (1/5 dose per mouse). A control group of mice were left un-primed. After three weeks, mice were immunized with 1 μ g A β _{1–6}-CMV_{TT} or A β _{1–6}-CMV_{WT} vaccine intravenously. Blood was collected on days 0, 3, 7 and 14 and analyzed for serum A β specific IgG antibodies as described previously. The antibody titers is reported as OD50 which is the reciprocal of the dilution which reaches half of the maximal OD value.

Immunohistology of Alzheimer's patients' brain sections

Sections of paraffin embedded brain (hippocampus) tissue of an Alzheimer's patient were prepared with a microtome. After deparaffinising, sections were blocked in BSA with normal horse serum. A monoclonal antibody raised against A β _{1–17} (Abcam ab11132), or by Protein G-purified mouse serum from day 0 (pre-immune) or day 56 (post-immune) after one immunization were applied to sections for 1 h at RT. To detect specific binding all pre-treated brain sections were incubated with a biotinylated horse anti-mouse IgG (Vectorlabs BA2000) for 30 min at RT, first and next, incubated with streptavidin labeled with AP. Binding was visualized by substrate Fast Red from Sigma. Subsequently, in order to visualize nuclei a counterstain using hematoxylin was performed. Stained slides were digitalized at 0.25 μ m per pixel resolution using a ScanScope XT (Aperio, Vista, CA, USA). Full-slide scans were stored as high-resolution (0.21 microns/pixel). Regions of interest were processed using ImageScope software (Aperio).

Data availability statement

The cryo-electron microscopy density map for CMV-tt VLP structures have deposited with the Electron Microscopy Data Bank: EMD-3855 and associated atomic coordinates submitted to the Protein Data Bank with accession number PDB: 5OW6.

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AUTHOR CONTRIBUTIONS

AZ: Performed and supervised the experiments. JW: performed the experiments and edited the manuscript. FZ: performed the experiments and edited the manuscript. AeT: performed the experiments and edited the manuscript. GJ: supervised the experiments and edited the manuscript. IB: supervised the experiments. AK: performed the experiments. DS: supervised the experiments and edited the manuscript. FS: performed the experiments. PE: performed the experiments. MM: performed the experiments. SH: performed the experiments. JF: supervised the

experiments, designed the project and wrote the manuscript. MB: supervised the experiments, designed the project and wrote the manuscript.

ADDITIONAL INFORMATION

Supplementary information accompanies the paper on the *npj Vaccines* website (<https://doi.org/10.1038/s41541-017-0030-8>).

Competing interests: All authors listed as affiliated with Hypopet, Saiba, or HealVax, have their own shares or are paid employees of these companies engaged in commercial vaccine development using the technology described here. The remaining authors declare no competing financial interests.

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REFERENCES

- Plotkin, S. A., Orenstein, W. A. & Offit, P. A. *Vaccines*. 6th edn.
- Tissot, A. C., Maurer, P., Nussberger, J., Sabat, R. & Pfister, T. et al. Effect of immunization against angiotensin II with CYT006-AngQb on ambulatory blood pressure: a double-blind, randomized, placebo-controlled phase IIa study. *Lancet*. **371**, 821–827, [https://doi.org/10.1016/S0140-6736\(08\)60381-5](https://doi.org/10.1016/S0140-6736(08)60381-5) (2008).
- Ambuhl, P. M., Tissot, A. C., Fulurija, A., Maurer, P. & Nussberger, J. et al. A vaccine for hypertension based on virus-like particles: preclinical efficacy and phase I safety and immunogenicity. *J. Hypertens.* **25**, 63–72, <https://doi.org/10.1097/HJH.0b013e32800ff5d6> (2007).
- Wildbaum, G. & Karin, N. Augmentation of natural immunity to a pro-inflammatory cytokine (TNF-alpha) by targeted DNA vaccine confers long-lasting resistance to experimental autoimmune encephalomyelitis. *Gene Ther.* **6**, 1128–1138, <https://doi.org/10.1038/sj.gt.3300915> (1999).
- Zagury, D., Le Buanec, H., Mathian, A., Larcier, P. & Burnett, R. et al. IFNalpha kinoid vaccine-induced neutralizing antibodies prevent clinical manifestations in a lupus flare murine model. *Proc. Natl. Acad. Sci. USA* **106**, 5294–5299, <https://doi.org/10.1073/pnas.0900615106> (2009).
- Cavelti-Weder, C., Timper, K., Seelig, E., Keller, C. & Osranek, M. et al. Development of an interleukin-1beta vaccine in patients with type 2 diabetes. *Mol. Ther.* **24**, 1003–1012, <https://doi.org/10.1038/mt.2015.227> (2016).
- Jennings, G. T. & Bachmann, M. F. Immunodugs: therapeutic VLP-based vaccines for chronic diseases. *Annu. Rev. Pharmacol. Toxicol.* **49**, 303–326, <https://doi.org/10.1146/annurev-pharmtox-061008-103129> (2009).
- Vanrenterghem, Y., Waer, M., Roels, L., Coosemans, W. & Christaens, M. R. et al. A prospective, randomized trial of pretransplant blood transfusions in cadaver kidney transplant candidates. Ieuven collaborative group for transplantation. *Transpl. Int.* **7**, S243–246 (1994). Suppl 1.
- Poland, G. A., Ovsyannikova, I. G., Jacobson, R. M., Vierkant, R. A. & Jacobsen, S. J. et al. Identification of an association between HLA class II alleles and low antibody levels after measles immunization. *Vaccine*. **20**, 430–438 (2001).
- Goncalves, L., Albarran, B., Salmen, S., Borges, L. & Fields, H. et al. The non-response to hepatitis B vaccination is associated with impaired lymphocyte activation. *Virology*. **326**, 20–28, <https://doi.org/10.1016/j.virol.2004.04.042> (2004).
- Klimek, L., Bachmann, M. F., Senti, G. & Kundig, T. M. Immunotherapy of type-1 allergies with virus-like particles and CpG-motifs. *Expert. Rev. Clin. Immunol.* **10**, 1059–1067, <https://doi.org/10.1586/1744666X.2014.924854> (2014).
- Jennings, G. T. & Bachmann, M. F. The coming of age of virus-like particle vaccines. *Biol. Chem.* **389**, 521–536 (2008).
- Panina-Bordignon, P., Tan, A., Termijtelen, A., Demotz, S. & Corradin, G. et al. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur. J. Immunol.* **19**, 2237–2242, <https://doi.org/10.1002/eji.1830191209> (1989).
- Valmori, D., Pessi, A., Bianchi, E. & Corradin, G. Use of human universally antigenic tetanus toxin T cell epitopes as carriers for human vaccination. *J. Immunol.* **149**, 717–721 (1992).
- Safety and Immunogenicity of Plant-Derived Pfs25 VLP-FhCMB Malaria Transmission Blocking Vaccine in Healthy Adults*, <https://clinicaltrials.gov/ct2/show/NCT02013687> (2017).
- Gellert, A., Salanki, K., Tombacz, K., Tuboly, T. & Balazs, E. A cucumber mosaic virus based expression system for the production of porcine circovirus specific vaccines. *PLoS ONE*. **7**, e26888, <https://doi.org/10.1371/journal.pone.0052688> (2012).
- Bessa, J., Schmitz, N., Hinton, H. J., Schwarz, K. & Jegerlehner, A. et al. Efficient induction of mucosal and systemic immune responses by virus-like particles administered intranasally: implications for vaccine design. *Eur. J. Immunol.* **38**, 114–126, <https://doi.org/10.1002/eji.200636959> (2008).
- Bessa, J., Jegerlehner, A., Hinton, H. J., Pumpens, P. & Saudan, P. et al. Alveolar macrophages and lung dendritic cells sense RNA and drive mucosal IgA

- responses. *J. Immunol.* **183**, 3788–3799, <https://doi.org/10.4049/jimmunol.0804004> (2009).
19. Farahnik, B., Beroukhi, K., Nakamura, M., Abrouk, M. & Zhu, T. H. et al. Anti-IL-17 agents for psoriasis: a review of phase III data. *J. Drugs. Dermatol.* **15**, 311–316 (2016).
20. Foerster, J. & Bachman, M. Beyond passive immunization: toward a nanoparticle-based IL-17 vaccine as first in class of future immune treatments. *Nanomedicine* **10**, 1361–1369, <https://doi.org/10.2217/nnm.14.215> (2015).
21. Rohn, T. A., Jennings, G. T., Hernandez, M., Grest, P. & Beck, M. et al. Vaccination against IL-17 suppresses autoimmune arthritis and encephalomyelitis. *Eur. J. Immunol.* **36**, 2857–2867, <https://doi.org/10.1002/eji.200636658> (2006).
22. Ha, H. L., Wang, H., Pisitkun, P., Kim, J. C. & Tassi, I. et al. IL-17 drives psoriatic inflammation via distinct, target cell-specific mechanisms. *Proc. Natl. Acad. Sci. USA* **111**, E3422–3431, <https://doi.org/10.1073/pnas.1400513111> (2014).
23. Ichikawa, K., Iwasaki, E., Baba, M. & Chapman, M. D. High prevalence of sensitization to cat allergen among Japanese children with asthma, living without cats. *Clin. Exp. Allergy.* **29**, 754–761 (1999).
24. Gronlund, H., Saarne, T., Gafvelin, G. & van Hage, M. The major cat allergen, Fel d 1, in diagnosis and therapy. *Int. Arch. Allerg. Immunol.* **151**, 265–274, <https://doi.org/10.1159/000250435> (2010).
25. Duffort, O. A., Carreira, J., Nitti, G., Polo, F. & Lombardero, M. Studies on the biochemical structure of the major cat allergen *Felis domesticus* I. *Mol. Immunol.* **28**, 301–309 (1991).
26. Schmitz, N., Dietmeier, K., Bauer, M., Maudrich, M. & Utzinger, S. et al. Displaying Fel d1 on virus-like particles prevents reactogenicity despite greatly enhanced immunogenicity: a novel therapy for cat allergy. *J. Exp. Med.* **206**, 1941–1955, <https://doi.org/10.1084/jem.20090199> (2009).
27. Doody, R. S., Thomas, R. G., Farlow, M., Iwatsubo, T. & Vellas, B. et al. Phase 3 trials of solanezumab for mild-to-moderate Alzheimer's disease. *N. Engl. J. Med.* **370**, 311–321, <https://doi.org/10.1056/NEJMoa1312889> (2014).
28. Sevigny, J., Chiao, P., Bussiere, T., Weinreb, P. H. & Williams, L. et al. The antibody aducanumab reduces Aβ plaques in Alzheimer's disease. *Nature*. **537**, 50–56, <https://doi.org/10.1038/nature19323> (2016).
29. Griffith, I. J., Craig, S., Pollock, J., Yu, X. B. & Morgenstern, J. P. et al. Expression and genomic structure of the genes encoding Fd1, the major allergen from the domestic cat. *Gene*. **113**, 263–268 (1992).
30. Morgenstern, J. P., Griffith, I. J., Brauer, A. W., Rogers, B. L. & Bond, J. F. et al. Amino acid sequence of Fel d1, the major allergen of the domestic cat: protein sequence analysis and cDNA cloning. *Proc. Natl. Acad. Sci. USA* **88**, 9690–9694 (1991).
31. Schober, L. J., Khandoga, A. L., Penz, S. M. & Siess, W. The EP3-agonist sulprostone, but not prostaglandin E2 potentiates platelet aggregation in human blood. *Thromb. Haemost.* **103**, 1268–1269, <https://doi.org/10.1160/TH09-12-0815> (2010).



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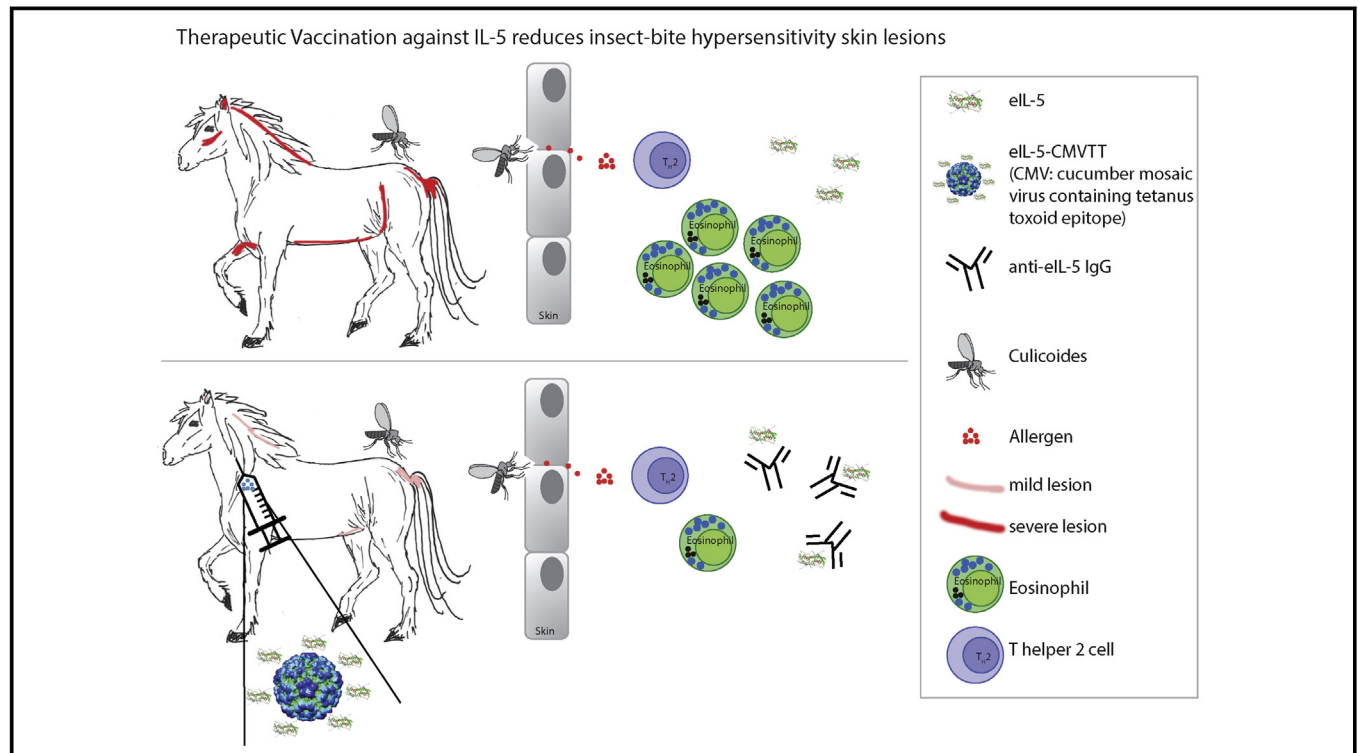
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Treating insect-bite hypersensitivity in horses with active vaccination against IL-5



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GRAPHICAL ABSTRACT



Background: Insect-bite hypersensitivity is the most common allergic dermatitis in horses. Excoriated skin lesions are typical symptoms of this seasonal and refractory chronic disease. On a

cellular level, the skin lesions are characterized by massive eosinophil infiltration caused by an underlying allergic response.

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Objective: To target these cells and treat disease, we developed a therapeutic vaccine against equine IL-5 (eIL-5), the master regulator of eosinophils.

Methods: The vaccine consisted of eIL-5 covalently linked to a virus-like particle derived from cucumber mosaic virus containing the tetanus toxoid universal T-cell epitope tt830-843 (CMV_{TT}). Thirty-four Icelandic horses were recruited and immunized with 400 µg of eIL-5–CMV_{TT} formulated in PBS without adjuvant (19 horses) or PBS alone (15 horses).

Results: The vaccine was well tolerated and did not reveal any safety concerns but was able to induce anti-eIL-5 autoantibody titers in 17 of 19 horses. This resulted in a statistically significant reduction in clinical lesion scores when compared with previous season levels, as well as levels in placebo-treated horses.

Protection required a minimal threshold of anti-eIL-5 antibodies. Clinical improvement by disease scoring showed that 47% and 21% of vaccinated horses reached 50% and 75% improvement, respectively. In the placebo group no horse reached 75% improvement, and only 13% reached 50% improvement.

Conclusion: Our therapeutic vaccine inducing autoantibodies against self IL-5 brings biologics to horses, is the first successful immunotherapeutic approach targeting a chronic disease in horses, and might facilitate development of a similar vaccine against IL-5 in human subjects. (J Allergy Clin Immunol 2018;142:1194-205.)

Key words: Allergic dermatitis, eosinophils, vaccination

Insect-bite hypersensitivity (IBH) of horses, also known as sweet itch, summer eczema, kasen, or Queensland itch, manifests as chronic relapsing seasonal allergic dermatitis caused by the biting of insects of the genus *Culicoides*.¹⁻⁵

Culicoides species midges are found in various areas of the world.⁶⁻⁸ The incidence of IBH correlates strongly with the geographic distribution of *Culicoides* species, showing the highest incidence in some parts of Australia (60%).⁹⁻¹¹ Overall, approximately 10% of all horses worldwide are affected by IBH,¹²⁻¹⁵ and in principle, all breeds can succumb to the allergic disease. During warmer months of the year, IBH-affected horses experience hairless, weeping, and sometimes even ulcerative lesions caused by inflammation and severe itching. Lesions are characterized by hyperkeratosis, lichenification of the skin, bleeding, swelling, scales, and crust formation. Histologic hallmarks of IBH lesions are thickening of the stratum corneum, epidermis, and dermis, with abundant fibrosis in the latter.⁹ Commonly, secondary infections with bacteria, mites, and fungi can cause further local irritation, enhancing lesion formation. Although IBH was first described in 1840 and is currently the best characterized allergic disease in horses, treatment options are still poor, and currently, no satisfactory treatment of IBH is available.^{9,16}

IBH is classified as IgE-dependent type I allergy,¹⁷ with a strong involvement of type IV allergic hypersensitivity reactions.^{4,18} In addition to IgE-mediated cross-linking of FcεRI on mast cells and basophils with subsequent histamine release, type I allergies also consist of a late-phase reaction with eosinophilia and recruitment of eosinophils into the allergic site. Intradermal injection of *Culicoides* species extract into IBH-affected horses leads to recruitment of T_H2 cells and

Abbreviations used

CMV:	Cucumber mosaic virus
CMV _{TT} :	Cucumber mosaic virus containing the tetanus toxoid universal T-cell epitope tt830-843
eIL-5:	Equine IL-5
HRP:	Horseradish peroxidase
IBH:	Insect-bite hypersensitivity
ISI:	Insect-bite hypersensitivity severity index
MALDI:	Matrix-assisted laser desorption ionization
MS/MS:	Tandem mass spectrometry
PBST:	PBS-Tween 0.1% (vol/vol)
VLP:	Virus-like particle

eosinophils to the injection site.¹⁹ Moreover, in addition to the role of IgE and type I allergy in the setting of IBH, an involvement of cell-mediated type IV allergic reactions, also called delayed-type hypersensitivity, has been discussed in recent years.¹⁶

Type IV allergies can be divided into 4 subgroups: (ie, types IVa, IVb, IVc, and IVd) depending on the cell types involved. Type IVb allergy is strongly associated with IL-5–producing T_H2 cells, eosinophilia, and eosinophilic type of inflammation²⁰ and reflects the clinical manifestations of skin inflammation in IBH, which are characterized by edema and eosinophil accumulation in perivascular clusters in deeper parts of the dermis.²¹ These enhanced eosinophils counts represent the predominant inflammatory cell type accumulating in IBH lesions.^{21,22} Activated eosinophils release granule enzymes and other effector molecules, such as histamine, eosinophil-derived neurotoxin, cysteinyl leukotrienes, and major basic protein. Major basic protein triggers degranulation of mast cells and basophils, thereby further enhancing allergic symptoms.²³ Cysteinyl leukotrienes derived from arachidonic acid are inflammatory mediators contributing on multiple levels to an allergic reaction, thereby making an eosinophil a very effective cell type to cause strong allergic symptoms during late-phase and delayed-type reactions. Therefore eosinophil degranulation can mediate hyperreactivity, inflammation, and local tissue damage, thus potentially driving the pathology of IBH.²⁴

To dampen eosinophil activity, we decided to target the lineage-specific eosinophil master regulator IL-5, which is a T_H2-type cytokine produced mainly by T_H2 cells and also by mast cells. Eosinophil development in the bone marrow is critically dependent on IL-5.^{25,26} Moreover, eosinophil release into blood circulation is mediated by IL-5, and the cytokine plays a key role in eosinophil activation.²⁷ Also, IL-5 increases survival of tissue-resident inflammatory eosinophils.²⁸ Mice either vaccinated against murine IL-5 or IL-5–deficient knockout mice show strongly reduced levels of blood eosinophils and eosinophil-mediated inflammation.^{26,29} Furthermore, mepolizumab, a humanized anti-IL-5 mAb, significantly reduced circulating eosinophil counts in human subjects and has shown clinical efficacy in patients with eosinophil-mediated diseases, such as eosinophil-mediated asthma and hypereosinophilic syndrome.³⁰⁻³⁴ Thus blocking IL-5 can lead to effective control of IBH.

Instead of targeting IL-5 with mAbs,³⁵ which is not a realistic approach because of the weight of a horse, we developed a

virus-like particle (VLP)-based therapeutic vaccine that induces IL-5 autoantibodies in the host. VLPs are supramolecular structures composed of multiple copies of 1 (or more) recombinantly expressed structural viral proteins, which spontaneously assemble into capsid particles but lack genetic information and therefore do not have replicative capacity, which is an important safety aspect. The highly repetitive surface, particulate structure, and induction of innate immunity through pathogen-associated molecular patterns is responsible for induction of fulminant B- and T-cell responses.^{36,37} VLPs can be derived from a large number of viruses, including bacteriophages and plant viruses,^{38,39} and are used for the development of a wide range of different prophylactic and therapeutic vaccines.

Here we used a recently developed and novel VLP platform based on the cucumber mosaic virus containing the tetanus toxoid universal T-cell epitope tt830-843 (CMV_{TT}) to enhance T_H cell-dependent IgG responses for antigens displayed on the VLP surface.⁴⁰ We show that a vaccine consisting of equine IL-5 (eIL-5) chemically linked to CMV_{TT}-VLPs induces potent anti-eIL-5 antibody responses, which improve symptoms of IBH in horses.

METHODS

Horses and clinical study

All study horses were Icelandic horses and privately held by their owners. All clinical studies were approved by the respective cantonal veterinary authorities. All horse owners signed informed consent. The clinical study was performed as a double-blind, placebo-controlled randomized trial; neither the person that performed the clinical trial nor the horse owners knew the group identity of the horses. The independent vaccine filler has randomized the study and was not involved in any clinical part of the study.

IBH lesion scoring

For IBH symptom scoring, all locations (tail, mane, belly, flank, face, ear, leg, and the like) at which IBH lesions occurred were recorded. Each location was divided into 3 parts: up, middle, and down. Furthermore, according to the number of lesions, each location was classified as light or strong, depending on the frequency of lesions within the particular area. Depending on how many parts are affected (up/middle/down) and how many lesions per location were found (light/strong), 1 to 4 points were scored (1 point = one part affected, lesion light; 4 points = all 3 parts affected, lesion strong). Moreover, these locations were classified for 6 further properties: size (diameter), blood (occurrence of bleeding), hair loss, scales, crust, and lichenification/swelling. For all these properties, 1 to 4 points were also scored. Lesion size was divided into 1 point (<0.5 cm), 2 points (0.5 ≤ < 1 cm), 3 points (1 ≤ < 2 cm), and 4 points (≥2 cm). The "blood" criteria served to distinguish between intact epidermis (1 point) or whether mild (2 points), moderate (3 points), or severe (4 points) bleeding occurred. Hair loss was divided into mild (1 point), moderate (2 points), severe (3 points), and absence of hair (4 points). Scales were divided into none (1 point), tiny and few (2 points), moderate and mid-sized (3 points), and many and big (4 points) scales. Crust was divided into none (1 point), tiny (2 points), half (3 points), and total (4 points). Lichenification and/or swelling were divided into none (1 point), mild (2 points), moderate (3 points), and severe (4 points). Additionally, if the sheath or udder was swollen, a minimum of 5 or a maximum of 20 points were scored: grade 1 (5 points), grade 2 (10 points), grade 3 (15 points), and grade 4 (20 points). Finally, all points were summed to produce the IBH symptom (or lesion) score.

The Δ average lesion score was calculated for placebo-treated and vaccinated horses as follows. Lesion scores for all horses were recorded monthly during the observation period within each IBH season. For each horse, the average lesion score was calculated per season. The Δ average lesion score was defined by subtracting the pretreatment season average lesion

score by the treatment season average lesion score. The average of the resulting differences was calculated and is reported here as Δ mean average lesion score.

Blood withdrawal

Blood was collected from the jugular vein at the intersection of the proximal to median third of the neck.

Blood analysis with IDEXX Diavet and production of horse sera and plasma

For differential blood analysis and determination of IgE levels against *Culicoides* species, blood was collected in tubes provided by IDEXX Diavet (Freienbach, Switzerland). Differential blood analysis was done with fresh EDTA blood. IgE levels against insects were determined in serum, EDTA plasma, and Lithium Heparin plasma; tubes were centrifuged at 3000 rpm for 10 minutes, and serum or plasma was transferred into fresh tubes. IgE-specific *Culicoides* species ELISA was performed by IDEXX Diavet with *Culicoides* species whole-body extract.

Detection of recombinant eIL-5 by anti-IL-5 ELISA

Maxisorp 96-well ELISA plates (Nunc; Thermo Fisher) were coated overnight with 50 μ L of anti-His antibody at 0.5 mg/L. Plates were washed 3 times with PBS-Tween 0.1% (vol/vol; PBST) and then blocked with Superblock (Thermo Scientific, Waltham, Mass) for 1 hour at 37°C. Plates were washed twice with PBST, and purified recombinant eIL-5-C-His was titrated starting with addition of 10 mg/L diluted 1:3 and incubated for 1 hour. Blank wells contain Superblock without recombinant protein. Then plates were washed 3 times with PBST and 1 ng/mL anti-eIL-5 antibody (eIL-5 affinity purified polyclonal antibody, catalog no. AF2470; R&D Systems, Abingdon, United Kingdom) was added and incubated at room temperature for 2 hours. Plates were washed subsequently 3 times with PBST and incubated with a secondary anti-goat IgG conjugated with horseradish peroxidase (HRP; dilution 1:2000) at room temperature for 30 minutes. Plates were again washed 3 times with PBS, and 50 μ L/well developing solution (TMB) was added. After 2 minutes of reaction at room temperature, the ELISA was stopped with 25 μ L/well at 5% H₂SO₄. Absorbance was measured at 450 nm on a Tecan M200 spectrophotometer (Tecan, Grödig, Austria).

eIL-5 levels in horse serum

ELISA was conducted according to the manufacturer's manual (R&D Systems).

Anti-CMV_{TT} and anti-IL-5 antibody titer determination

Maxisorp 96-well ELISA plates (Nunc) were coated overnight with purified eIL-5-C-His or purified CMV (5 g/L). Plates were washed 3 times with PBST and blocked with Superblock (Thermo Scientific) for 2 hours at room temperature. Then plates were washed 3 times with PBST, and 3-fold dilutions of 1:10 diluted horse sera (in Superblock) were added and incubated at room temperature for 2 hours. Plates were washed subsequently 3 times with PBST and incubated with anti-equine IgG (1:2000) conjugated with HRP (Jackson ImmunoResearch, West Grove, Pa) at room temperature for 30 minutes. Plates were again washed 4 times with PBS, and developing solution (TMB) was added. After approximately 2 minutes of reaction at room temperature, the ELISA was stopped with 5% H₂SO₄. Absorbance was measured at 450 nm (OD₄₅₀) on a Tecan M200 spectrophotometer (Tecan). The antibody titer as OD₅₀ was calculated (serum dilution on a logarithmic scale where OD₄₅₀ was half maximal). All antibody titers are calculated with naive serum subtracted on logarithmic scales and presented as Δ OD₅₀ values. Titers of less than 10 (and including 10) were considered as background.

IBH lesion biopsy and hematoxylin and eosin staining

Punch biopsy specimens (6 mm) from lesions of IBH-affected horses were stained with hematoxylin and eosin and CD3 (Vetsuisse Faculty, Zurich, Switzerland).

Cloning, expression, and purification of recombinant eIL-5

The DNA sequence encoding mature eIL-5 (*Equus caballus*; UniProt O02699) was generated by means of gene synthesis. In addition, a 3-amino-acid linker (GGC) was added C-terminally. This insert was flanked by 5' *NdeI* and 3' *XhoI* and integrated into pET 42b (+), containing a hexa His-tag (to facilitate purification) and an in-frame stop codon. The resulting eIL-5 fusion protein was expressed in *Escherichia coli* BL21 (DE3) cells. Overnight cultures were grown and diluted in LB medium containing 50 mg/L kanamycin. Isopropyl- β -D-thiogalactopyranosid was added to a final concentration of 1 mmol/L when an OD₆₀₀ of culture reached 0.7. The culture was harvested by means of centrifugation 4 hours after induction, and the pellet was resuspended in 100 mmol/L Tris/HCl (pH 8.0) at 4°C and 1 mmol/L EDTA. Inclusion bodies were prepared, and insoluble eIL-5 was solubilized in denaturing buffer (6 mol/L GdmCl, 20 mmol/L imidazole, and 100 mmol/L Tris-HCl [pH 8]). After centrifugation for 20 minutes at 20,000g, the supernatant containing denatured, soluble, recombinant eIL-5 was purified through the His-tag by using Ni-NTA (Ni-NTA Sepharose 6 Fast Flow, Amersham, Piscataway, NJ, or NiNTA Superflow, Qiagen, Hilden, Germany) column and eluted by using 6 mol/L GdmCl, 100 mmol/L NaH₂PO₄, and 10 mmol/L Tris-HCl (pH 4.5) with subsequent dialysis to pH 8.0. Accordingly, eIL-5 was refolded by means of sequential dialysis against the following buffers at pH 8.5 at 4°C: buffer 1 (2 mol/L urea, 50 mmol/L NaH₂PO₄, 5 mmol/L reduced glutathione, 0.5 mmol/L oxidized glutathione, 0.5 mol/L arginine, and 10% glycerol), buffer 2 (50 mmol/L NaH₂PO₄, 5 mmol/L reduced glutathione, 0.5 mmol/L oxidized glutathione, 0.5 mol/L arginine, and 10% glycerol), buffer 3 (50 mmol/L NaH₂PO₄ and 10% glycerol), and buffer 4 (PBS). Finally, refolded protein was concentrated by using Amicon Centrifugal Filters (Ultrafree-15 [Millipore, Temecula, Calif], 10 kDa MWCO) and purified on a HiLoad 26/60 Superdex 75 prep grade (GE Healthcare, Fairfield, Conn) with PBS buffer. Protein concentrations were determined by using the Bradford assay to BSA standard.

Circular dichroism spectroscopy of purified eIL-5-C-His

The far-UV circular dichroism spectrum of purified eIL-5-C-His (in PBS) was measured on a J-710 spectropolarimeter (Jasco, Easton, Md) at 25°C by using a 1-mm cuvette. After correction for the buffer spectrum, ellipticity was converted to mean residue ellipticity, as previously described.⁴¹

Coupling of eIL-5 to CMV_{TT}

VLP-CMV_{TT} derived from CMV and containing the tetanus toxoid universal T-cell epitope tt830-843 was expressed in *Escherichia coli* by using the expression plasmid pET28e, as previously described.⁴⁰ The CMV concentration was determined by using the Bradford assay to a BSA standard. CMV was reacted with a 10-fold molar excess of the heterobifunctional cross-linker succinimidyl-6(β -maleimidopropionamido)hexanoate in 20 mmol/L NaP/2 mmol/L EDTA, pH 7.5, at 25°C (Pierce, Rockford, Ill). Unreacted cross-linker was removed by means of passage over a PD-10 desalting column (GE Healthcare). Recombinant, purified, and refolded eIL-5-C-His was reduced for 1 hour with an equimolar amount of tri(2-carboxyethyl)phosphine hydrochloride in PBS (pH 8.0) to reduce the cysteine residue contained in the linker. The reduced eIL-5-C-His was then mixed with the derivatized CMV_{TT} VLPs at a molar ratio of CMV_{TT} monomer to eIL-5-C-His protein monomer of 1:2 and coincubated for 4 hours at 22°C in PBS (pH 7.4) to allow cross-linking.

SDS-PAGE and Coomassie staining

Separation of proteins with 4–12% Bis-Tris SDS-PAGE gels was done with a prestained SeeBlue marker (Thermo Fisher) and MES buffer (NuPAGE; Novex, Invitrogen Life Technologies, Grand Island, NY) at 200 V for 55 minutes. For coupling analysis, equimolar amounts of CMV_{TT}, eIL-5-C-His, and eIL-5-C-His-CMV_{TT} VLPs were separated by using SDS-PAGE, 4–12% B/T gel (NuPAGE, Invitrogen), and MES buffer at 200 V for 55 minutes. Subsequently, the gel was stained with Coomassie Blue (0.025% Coomassie Brilliant Blue R-250, 40% methanol, and 10% acetic acid) and destained with destainer (40% methanol and 10% acetic acid). The coupling efficiency (ie, moles of CMV-IL-5/moles of CMV monomer [total]) was estimated by using densitometric analysis of the Coomassie Blue-stained SDS-PAGE with ImageJ software (National Institutes of Health, Bethesda, Md).

Western blotting

CMV_{TT}, eIL-5-C-His, and IL-5-C-His-CMV_{TT} vaccine were separated by using SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was blocked for 1 hour with 5% (wt/vol) BSA powder in PBST and then incubated with 10 mL of 1:1000 diluted anti-His antibody (Penta-His Antibody, BSA-free, mouse monoclonal IgG₁; Qiagen) in 1% BSA (wt/vol) powder in PBST. The membrane was washed with PBST for 15 minutes and then incubated for 1 hour with 10 mL of 1% (wt/vol) BSA in PBST containing an anti-mouse IgG antibody conjugated with HRP at a dilution of 1:10,000. The membrane was washed for 15 minutes in PBS and developed with ECL (Amersham Pharmacia, Sweden) and exposed to photographic film.

Vaccine administration and immunization regimen

Horses (n = 19) were injected subcutaneously on days 0, 28, 56, and 84 with 400 μ g of eIL-5-C-His-CMV_{TT} VLPs in 1000 μ L of PBS without additional adjuvants to generate self-reactive antibodies to eIL-5. All horses received a booster immunization on day 140. Placebo-treated horses (n = 15) received PBS instead at all respective time points.

Parasite load

Fresh feces was collected from horses before and after vaccination and was analyzed at IDEXX Diavet by using the combined sediment/float method, distinguishing gastrointestinal nematodes, coccidian oocysts, and tapeworm eggs/body parts. For each time point (before and after vaccination), a value of 1 was allocated to horses with helminths, and a value of 0 was allocated to horses without helminths. Mean helminth values were calculated for eIL-5-CMV_{TT}-vaccinated and placebo-treated horses. The Δ helminth load for placebo-treated or vaccinated horses was calculated by subtracting the mean value after treatment from the mean value before treatment.

Statistics

All graphs comparing vaccinated horses versus placebo-treated horses show means and SEMs. For statistical analysis, vaccine horses versus placebo horses were compared by using an unpaired 2-tailed Student *t* test. *P* values of less than .05 were considered to be statistically significant.

RESULTS

Eosinophil counts but not IgE levels correlate with disease severity

To compare the influence of levels of *Culicoides* species-specific IgE versus eosinophil counts, we clinically scored signs of IBH (by score sheet, see Fig E1 in this article's Online Repository at www.jacionline.org) in affected horses for a whole season and analyzed blood for blood eosinophil counts and *Culicoides* species-specific IgE levels using a commercial serologic test. Blood was taken in the season before vaccination. Eleven of the

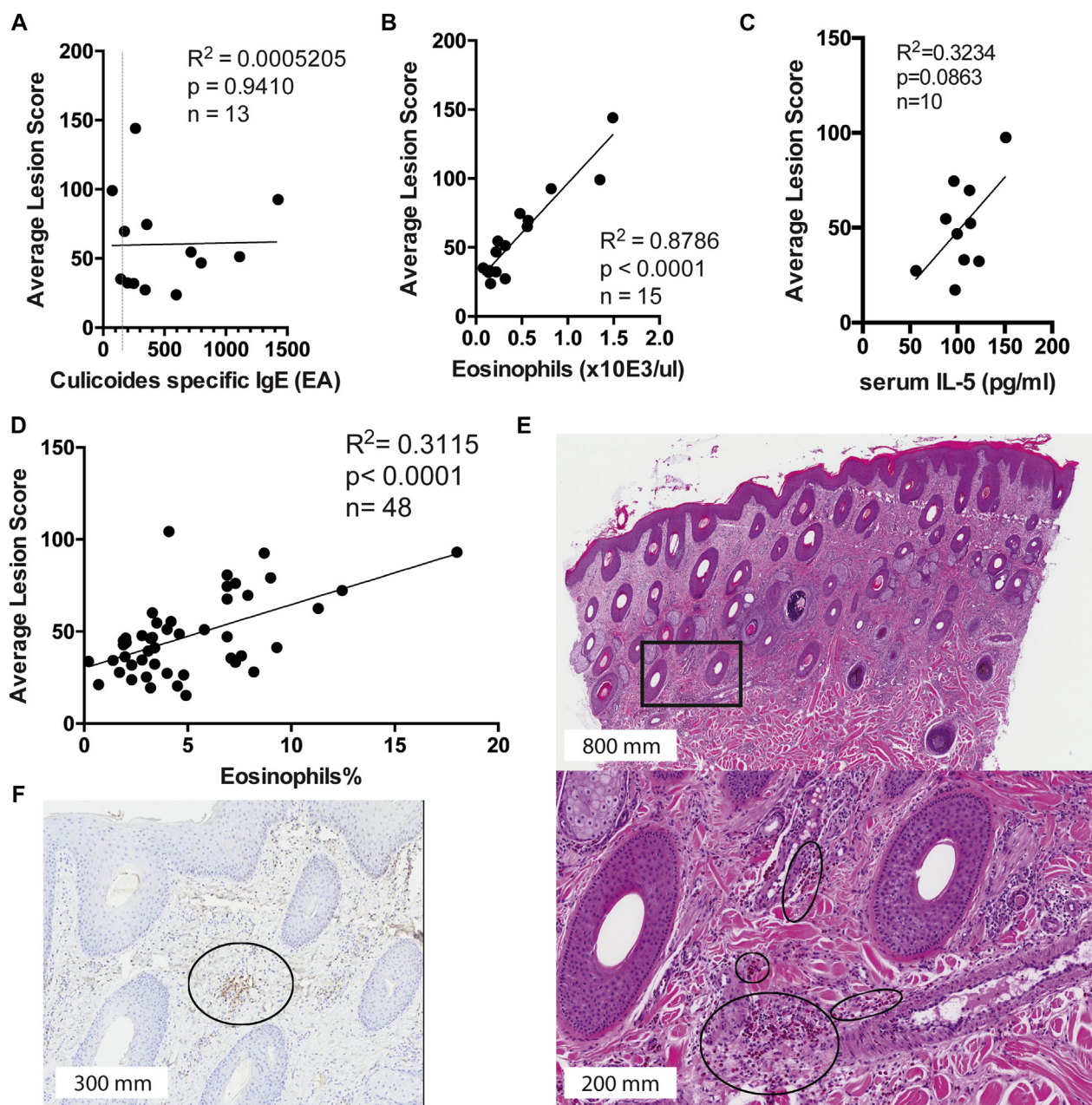


FIG 1. Diagnostics and characterization of IBH. **A–D**, Correlation of IBH lesion scores to blood parameters. IBH lesion scores were determined in biweekly to 4-week intervals during a whole season (April until October). Average lesion scores per horse were generated. EDTA blood and serum were taken once during the season with high symptoms (August). Eosinophil levels (EDTA blood) and *Culicoides* species-specific IgE levels (serum) were quantified by IDEXX Diavet. Correlation between average lesion scores and *Culicoides* species-specific IgE levels (IgE levels >150 ELISA absorbance units [EA] are considered allergic; dotted gray line; Fig 1, A), blood eosinophil count (Fig 1, B), serum IL-5 levels (quantified by means of ELISA; Fig 1, C), and blood eosinophil counts in a larger cohort (Fig 1, D) are shown. **E** and **F**, IBH skin biopsy. A representative skin biopsy specimen of a fresh lesion from an IBH-affected horse. Fig 1, E, Eosinophils in an IBH skin biopsy specimen. Hematoxylin and eosin staining was used. An overview (scale bar = 800 μm , upper panel) and enlarged section (scale bar = 200 μm , lower panel) are shown. Eosinophil-rich regions in the enlarged section are marked by circles. Fig 1, F, IBH skin biopsy specimen. CD3 staining was used. An overview (scale bar = 300 μm) is shown. A CD3⁺-rich region is marked by a circle.

13 tested IBH-affected horses had *Culicoides* species-specific IgE levels of greater than the cutoff of 150 ELISA absorbance units. However, 1 horse with very severe clinical signs of IBH (average IBH score, 99) had levels less than the cutoff, and

overall, IgE levels did not correlate with average scores of seasonal IBH severity (Fig 1, A). In contrast, there was a strong positive correlation between average seasonal IBH scores and eosinophil count in blood at time points of active disease

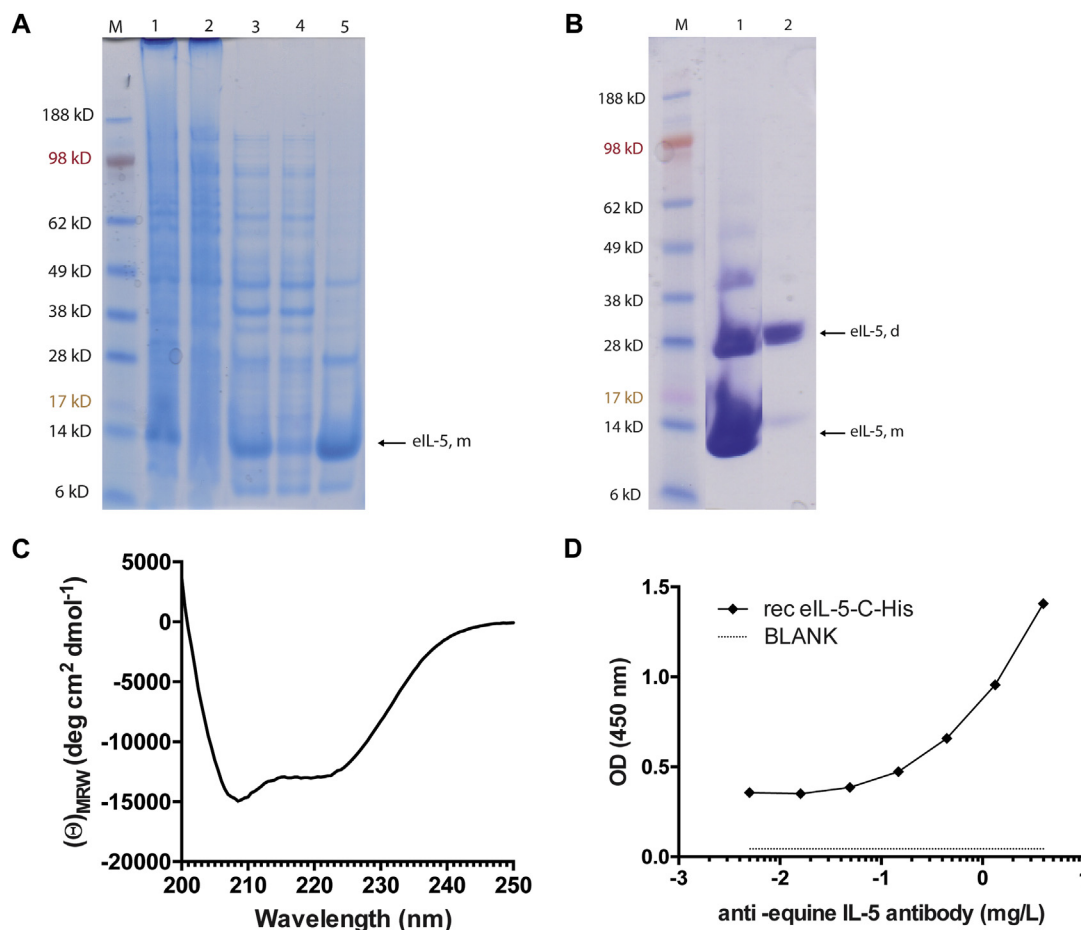


FIG 2. Production and characterization of eIL-5 protein. **A**, SDS-PAGE analysis of purified eIL-5-C-His. Samples from various stages of the inclusion body preparation and purification were applied to a 4-12% B/T gel and run under reducing conditions. Proteins were stained with Coomassie Blue. *Lane M*, Size marker; *lane 1*, lysate (sample A); *lane 2*, soluble fraction (sample B); *lane 3*, solubilized inclusion bodies (sample C); *lane 4*, flow through (unbound material, sample D); and *lane 5*, pooled eIL-5 monomer (eIL-5, m) eluate from the Ni-NTA column (sample E). **B**, SDS-PAGE of refolded recombinant eIL-5-C-His. An aliquot of purified eIL-5-C-His was separated on a 4-12% B/T gel and run under nonreducing conditions (+SDS, no dithiothreitol, no heating). Proteins were stained with Coomassie Blue. eIL-5, d, eIL-5 dimer; eIL-5, m, eIL-5 monomer. *Lane M*, Size marker; *lane 1*, pooled denatured eluate from the Ni-NTA column; *lane 2*, refolded and homodimer enriched eIL-5-C-His. **C**, Far-UV circular dichroism spectrum of purified eIL-5-C-His. The spectrum demonstrates that eIL-5-C-His was folded. **D**, Detection of purified eIL-5-C-His with commercial anti-eIL-5 antibody. Anti-His antibody-coated ELISA plates were incubated with purified eIL-5-C-His and detected with a commercially available anti-eIL-5 antibody.

(Fig 1, B). Moreover, serum IL-5 levels at the same time point also correlated to average seasonal IBH scores, thereby indicating a link between levels of eIL-5 and IBH-associated eosinophilia (Fig 1, C). The correlation of eosinophil blood counts with IBH scores was also seen when a second larger cohort of horses was assessed (Fig 1, D). A representative biopsy of a skin lesion stained with hematoxylin and eosin is shown in Fig 1, E, confirming recruitment of blood eosinophils to the skin. In addition to the remarkable eosinophil infiltrates, the most abundant other cell type also associated with skin pathology was the CD3⁺ T cell (Fig 1, F). Furthermore, the typical hyperkeratosis accompanied by acanthosis can be found, fulfilling the histologic hallmarks of an IBH lesion (Fig 1, E).

Taken together, blood eosinophil counts correlate strongly with IBH severity scores and therefore might not only be useful to monitor IBH severity but also are an attractive target in IBH

therapy. Consequently, a therapy treating eosinophilia is likely to influence the magnitude of disease severity.

Production of therapeutic vaccine

To induce autoantibodies against eIL-5, we coupled eIL-5 to VLPs, creating a hapten-carrier complex and thus bypassing B-cell unresponsiveness against self IL-5 by introducing VLP-specific intermolecular T-cell help.⁴²

Recombinant eIL-5 with a C-terminal linker containing a free cysteine residue and a His-tag (eIL-5-C-His) was produced in *E coli* and purified by means of affinity chromatography (Fig 2, A), refolded, and polished by using size-exclusion chromatography. Because biologically active native IL-5 is a disulfide-linked homodimer, the ability of purified recombinant eIL-5-C-His to form dimers after refolding was assessed by using

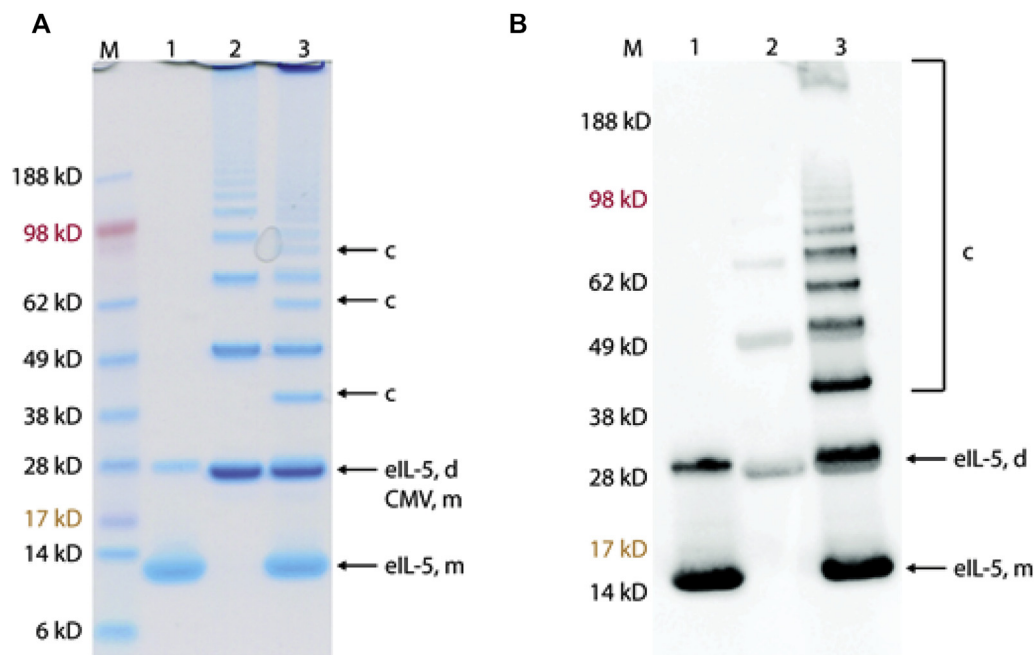


FIG 3. Vaccine production and analysis of the coupling reaction of eIL-5-C-His to CMV_{TT}. **A** and **B**: Lane M, Size marker; lane 1, tri(2-carboxyethyl)phosphine hydrochloride (TCEP)-activated eIL-5-C-His; lane 2, CMV-VLP after derivatization with the chemical cross-linker succinimidyl-6(β-maleimidopropionamido)hexanoate (SMPH); lane 3, eIL-5-C-His-CMV_{TT} coupling reaction. eIL-5-C-His, CMV_{TT}, and eIL-5-CMV_{TT} were loaded in an equimolar manner to compare coupling efficacy. **A**, SDS-PAGE. Proteins were stained with Coomassie Blue. c, Coupling; CMV, m, CMV_{TT} monomer; eIL-5, d, eIL-5 dimer; eIL-5, m, eIL-5 monomer. **B**, Western blotting with α-His antibody staining. c, Coupling; eIL-5, d, eIL-5 dimer; eIL-5, m, eIL-5 monomer.

SDS-PAGE performed under nonreducing conditions (Fig 2, B, lane 2). As judged by the molecular mass of approximately 28 kDa, eIL-5-C-His was indeed dimeric in nature, indicating conservation of the native tertiary structure. Far-UV circular dichroism spectroscopy confirmed that purified eIL-5-C-His was folded and, as expected and indicated by the minima at 208 and 222 nm, adopted a mostly α-helical secondary structure (Fig 2, C). Furthermore, a commercially available anti-eIL-5 mAb also recognized the refolded dimeric eIL-5 (Fig 2, D). In the native structure of the IL-5 dimer, the 2 monomers are linked by 2 intermolecular disulfide bridges (between Cys44 of one monomer and Cys86 of the other and *vice versa*), leading to a head-to-tail positioning of the 2 monomers with respect to each other.⁴³ In particular, linkage of 2 monomers by 2 intermolecular disulfide bridges was analyzed by using liquid chromatography–matrix-assisted laser desorption ionization (MALDI)–tandem mass spectrometry (MS/MS) after trypsin digestion of eIL-5-C-His. MS/MS fractions of mass 2505, 2633, and 2761 m/z showed the typical disulfide fragment pattern 32/2/32, confirming intermolecular linkage of 2 IL-5 monomers through Cys44 to Cys86 (see Fig E2 in this article's Online Repository at www.jacionline.org).

The eIL-5-C-His homodimers were chemically coupled to VLPs derived from the cucumber mosaic virus (CMV_{TT}) through the heterobifunctional cross-linker succinimidyl-6(β-maleimidopropionamido)hexanoate to produce a VLP-based vaccine against IL-5. Derivatization of VLPs shows the typical VLP ladder with CMV monomeric and multimeric subunits (Fig 3, A, lane 2).²⁹ Coupling of CMV subunits with dimeric eIL-5-C-His molecules (Fig 3, A, lane 1) is shown on a reducing SDS-PAGE gel by

additional coupling bands that correspond to the molecular mass of monomeric or dimeric eIL-5 plus monomeric or multimeric CMV subunits (Fig 3, A, lane 3). Successful covalent attachment of eIL-5-C-His to CMV_{TT} was confirmed by using Coomassie staining (Fig 3, A) and Western blotting with an anti-His antibody (Fig 3, B). Coupling efficiency for all batches was between 20% and 50%.

Antibody titer responses on vaccination

Horses were immunized with 400 μg of eIL-5-CMV_{TT} formulated in PBS on days 0, 28, 56, 84, and 140. Placebo-treated horses received PBS. The reasons to use PBS and not CMV_{TT} without IL-5 coupled to a placebo were 2-fold: (1) placebo in registration studies will be PBS and not CMV_{TT} without IL-5 coupled to it, and (2) we planned to immunize the placebo-treated horses in the following season, which would have been impossible if they had been immunized with CMV_{TT} alone because of carrier-induced epitope suppression.⁴⁴ Therefore we decided to use PBS as a control. Antibody titers in horse serum were evaluated monthly after vaccination and were consistently detectable after the second injection (Fig 4). The third and fourth injections maintained antibody responses against eIL-5 (Fig 4, A) and CMV_{TT} (Fig 4, B). Variation between single horses was less pronounced for anti-CMV_{TT} VLP antibody titers (Fig 4, D) than for antibody titers against the self-molecule eIL-5 (Fig 4, C). However, and in contrast to anti-CMV antibodies, anti-IL-5 antibodies can bind to their target antigen, IL-5, in serum. Hence such antigen-antibody complexes might not be detectable by using classical ELISA.

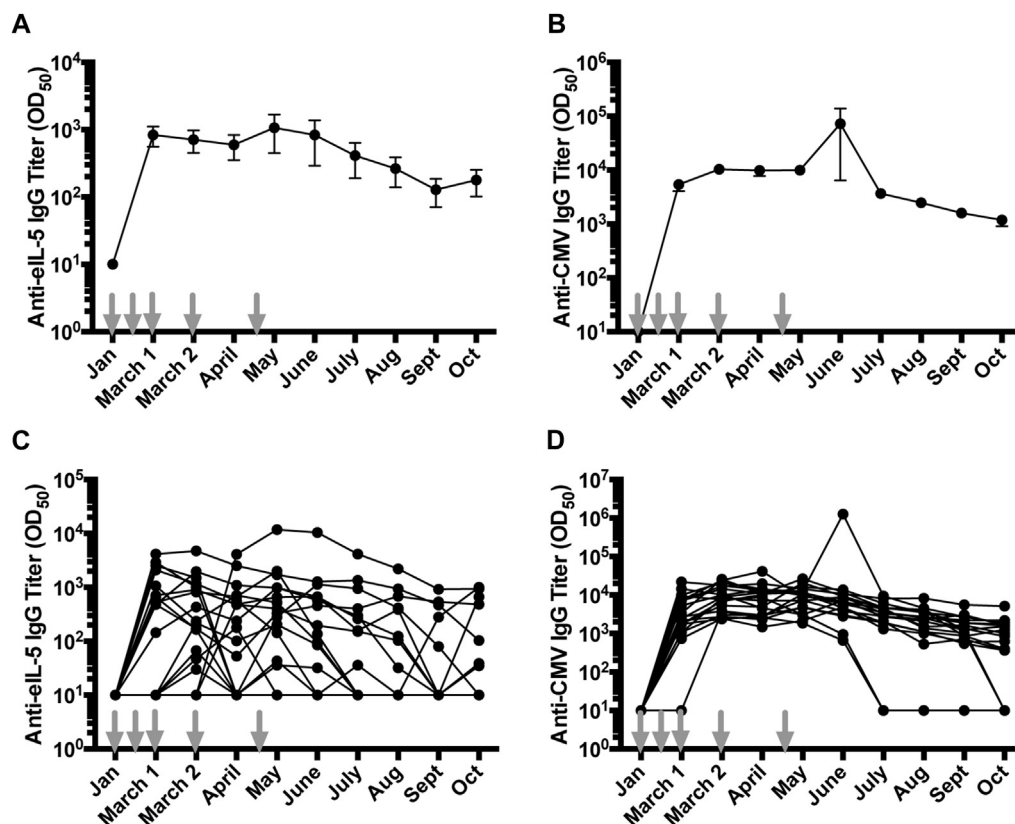


FIG 4. Antibody titer of vaccinated horses against eIL-5 and CMV_{TT}-VLP. Time points of vaccinations are indicated by gray arrows. **A**, Mean antibody titer of anti-eIL-5 IgG. **B**, Mean antibody titer of anti-CMV IgG. **C**, Antibody titer of anti-eIL-5 IgG of individual horses. **D**, Antibody titer of anti-CMV IgG of individual horses.

Mean antibody titers were decreasing toward the end of the season for both eIL-5 and CMV (Fig 4).

Reduced IBH lesion scores in eIL-5-CMV_{TT}-vaccinated horses

Lesion scores of study horses were recorded monthly in the pretreatment season and the subsequent treatment year from March until October. Data were analyzed according to an intention-to-treat analysis, thus including all horses without exclusions. Seasonal progression of average lesion scores of placebo-treated horses were comparable for both seasons (Fig 5, A), whereas average lesion scores of eIL-5-CMV_{TT}-vaccinated horses showed strongly improved clinical signs in the treatment year when compared with those in the pretreatment season (Fig 5, B). When comparing average lesion scores between both seasons for all horses individually, the majority of vaccinated horses showed pronounced lesion reduction in the treatment year. In contrast, most placebo-treated horses did not improve their symptoms (Fig 5, C). Analogous to Psoriasis Area and Severity Index 50 and Psoriasis Area and Severity Index 75 scores, we defined the insect-bite hypersensitivity severity index (ISI) 50 and ISI 75 (IBH severity index). ISI 50 represents a 50% and greater reduction of symptoms, and ISI 75 represents a 75% and greater reduction of symptoms. In total, 47% of vaccinated horses reached an ISI 50, whereas in the placebo group only 13% reached this level of reduction. An ISI 75 was achieved in

21% of the vaccinated horses, whereas none of the placebo-treated horses showed such a pronounced improvement (Fig 5, D). The Δ average lesion scores of eIL-5-CMV_{TT}-vaccinated horses showed a mean reduction in lesion scores of 28.5 ± 4.5 ($n = 19$), whereas the Δ average lesion score in placebo-treated horses showed a mean reduction in lesion score of 11.1 ± 3.7 ($n = 15$). The difference between vaccinated horses and placebo-treated horses was statistically significant, showing a higher improvement in lesion symptoms in eIL-5-CMV_{TT}-vaccinated horses ($P = .0073$; Fig 5, E). Furthermore, anti-eIL-5 serum antibody levels during the IBH season were most likely required to be greater than a certain threshold to be protective rather than showing a linear dependency (Fig 5, F). For further IBH vaccine development, the observed antibody threshold level can be used as a surrogate for clinical efficacy.

Blood eosinophilia

Eosinophil counts for all horses were measured monthly after vaccination or placebo treatment. During the IBH season, blood eosinophil counts of placebo-treated horses peaked in June, whereas lesion scores peaked with a delay of approximately 1 month in July. Mean blood eosinophil counts in placebo-treated horses had been found above maximal standard values from May until October, with a slight decrease in peak levels in July and October (Fig 6, A). In general, blood eosinophil counts of placebo-treated horses correlated well with lesion scores of the skin (Fig 6, B).

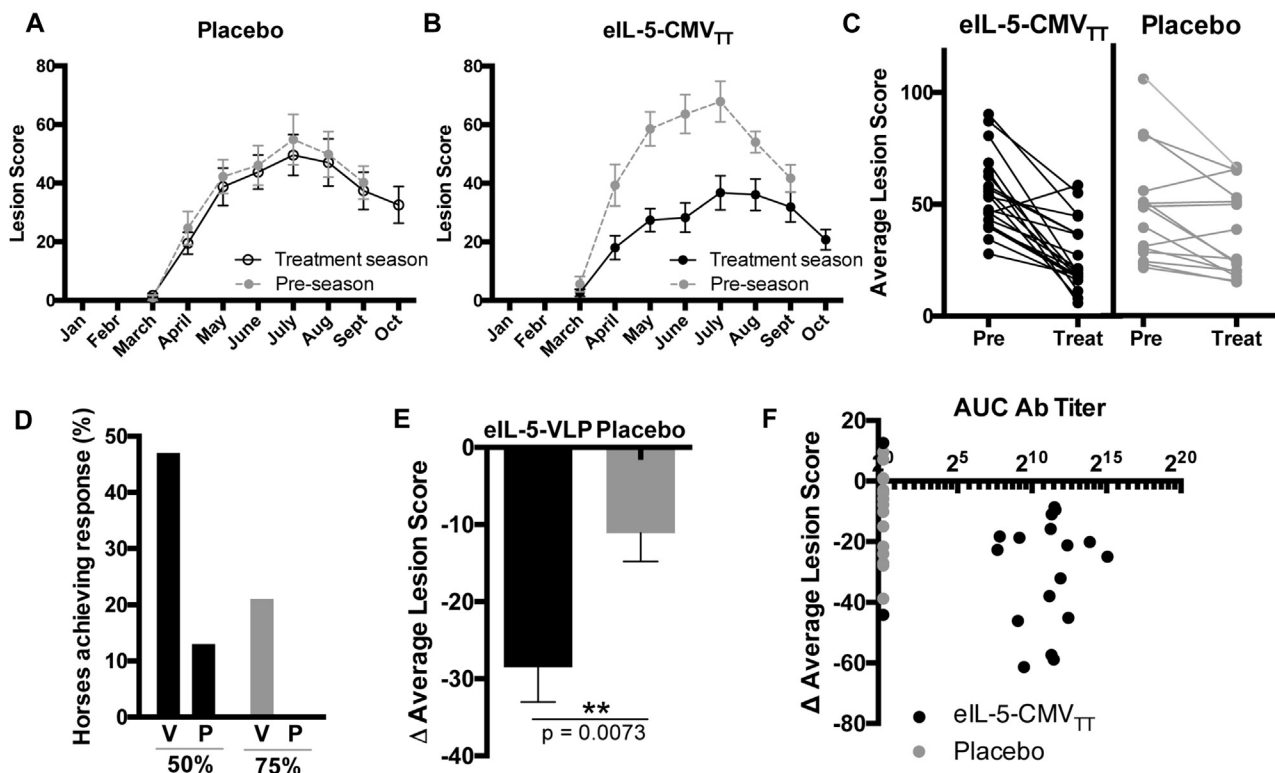


FIG 5. Lesion scores of eIL-5-CMV_{TT}-vaccinated horses improved significantly compared with placebo-treated horses in clinical study. **A** and **B**, Mean lesion score of placebo-treated horses ($n = 15$; Fig 5, **A**) and eIL-5-CMV_{TT}-vaccinated horses ($n = 19$; Fig 5, **B**) followed from March until October in the pre-evaluation year (gray symbols) and treatment year (black symbols). Months with more than 1 measurement show average values. **C**, Average lesion score per horse of the previous season (Pre) versus the treatment season (Treat; black: active, eIL-5-CMV_{TT} vaccinated, $n = 19$; gray: placebo, $n = 15$). **D**, Percentage of horses with 50% (black columns) and 75% (gray columns) lesion score improvement in eIL-5-CMV_{TT}-vaccinated (V) and placebo (P) group. **E**, Δ Mean average lesion score from eIL-5-CMV_{TT}-vaccinated horses (black column, $n = 19$) versus placebo-treated horses (gray column, $n = 15$). ** $P < .01$. **F**, Area under the curve (AUC) anti-eIL-5 antibody (Ab) titer versus Δ average lesion score of placebo-treated horses (gray symbols, $n = 15$) and eIL-5-CMV_{TT}-vaccinated horses (black symbols, $n = 19$). AUC includes all values during the whole treatment season. All graphs include all horses ($n = 34$) analyzed according to intention to treat.

Immunization against eIL-5 did not greatly alter blood eosinophil counts, and there was a much less obvious correlation between blood eosinophil count and lesion scores, indicating that anti-IL-5 antibodies alter eosinophil inflammation in lesions but had little influence on blood eosinophil counts (Fig 6, C).

No increase in parasitic load after eIL-5-CMV_{TT} vaccination

The parasitic load of horses was quantified in excrement at the start and end of the treatment year. No difference between placebo-treated and vaccinated animals was observed when comparing mean helminth load before and after treatment (Fig 7, A) and change in mean helminth load (Fig 7, B). In general, helminth burden in autumn was greater in both the placebo-treated and vaccinated groups, probably because of higher exposure to parasites on grazing land in the summer months.

DISCUSSION

IBH is a frequent and sometimes devastating disease in horses. Here we demonstrate that targeting eosinophils, the major

inflammatory cells in IBH lesions, with active vaccination against the T_H2 cytokine IL-5 strongly improves disease symptoms in affected horses. This is a novel and allergen-independent approach to manage IBH in horses that differs from the strategy of Jonsdottir et al,⁴⁵ who propose desensitizing horses through intralymphatic injection of small doses of allergens in adjuvants.

Despite a missing correlation between allergen-specific IgE levels and IBH severity, we showed that the severity of IBH disease correlated well with blood eosinophil counts. Especially in the setting of progressed IBH, eosinophils seemed to dominate the allergic reaction. The eosinophilia is caused by a late phase of type I and delayed-type allergic responses (type IV) to insect bites. This is in contrast to insect venom allergy in human subjects, in which eosinophilia has not been described. Thus eosinophil-dependent IBH shares common aspects with human asthma, which also has an IgE component but is eosinophil driven.⁴⁶ This implies that human allergy in the lungs seems to share common aspects with equine allergy in the skin. However, atopic dermatitis of human skin is a multifactorial and complex disease with various trigger factors, only one of them being allergens. Although serum concentration of human eosinophilic cationic protein is a diagnostic marker for atopic dermatitis in

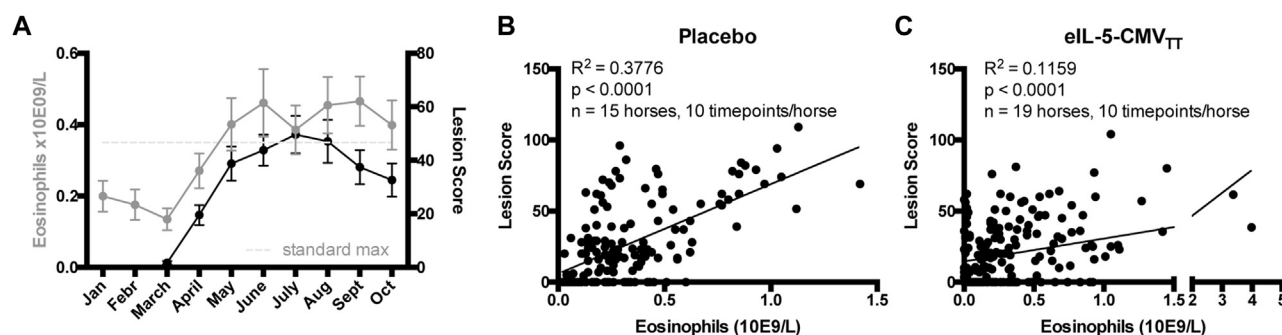


FIG 6. Interrelation between blood eosinophil counts and lesion scores. **A**, Mean blood eosinophil counts (gray symbols and line, left y-axis) and mean lesion scores (black symbol and line, right y-axis) of placebo-treated horses ($n = 15$) determined in the treatment season from January until October or March until October, respectively. The normal maximum blood eosinophil count is indicated by a dotted gray line. Months with more than 1 measurement show average lesion score values. **B**, Eosinophil counts versus lesion scores of all 15 placebo-treated horses for 10 time points from each horse. **C**, Eosinophil counts versus lesion scores of all 19 vaccinated horses for 10 time points from each horse.

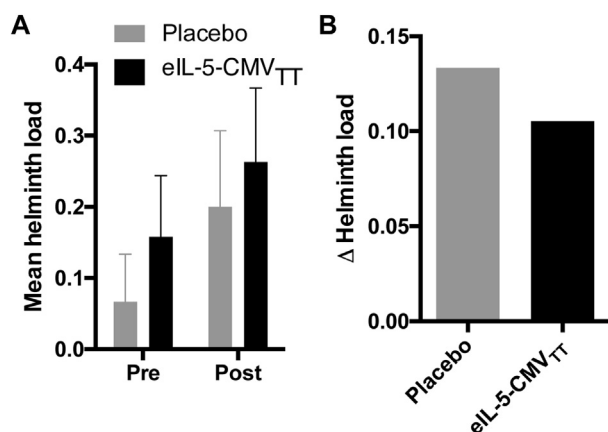


FIG 7. Side effect: parasitic load. **A**, Mean helminth burden in placebo-treated horses (gray columns, $n = 15$) and eIL-5-CMV_{TT}-vaccinated horses (black columns, $n = 19$). No measurable helminths in excrement = 0, detected helminths in excrement = 1. Excrement was taken in the treatment season before injections (pre) and at the study's end in autumn (post). **B**, Average seasonal change in helminth burden for placebo-treated (gray column) and vaccinated horses (black column) was calculated by subtracting the mean value determined at the beginning from that at the end of the treatment season.

human subjects,⁴⁷ eosinophilia is not suggested to trigger dermatitis in human subjects.⁴⁸ Therefore we think that human asthma is the correlate to equine IBH, although the affected organs differ.

IL-5 is a very well-validated target, and abundant data in mice and human subjects indicate that blocking IL-5 is a safe and efficacious way to reduce eosinophil-mediated inflammation. Therefore IL-5 might be an ideal target for safe IBH therapy in horses. Two different anti-human IL-5 antibodies have been developed for the treatment of eosinophil-associated diseases, such as hypereosinophilic syndrome or eosinophilic asthma: mepolizumab (GlaxoSmithKline, Research Triangle Park, NC) and reslizumab (Teva Pharmaceuticals, Petah Tikva, Israel). The mechanism of action for an anti-IL-5 antibody is blockage of eosinophil development, survival, and activation. Evidence from

eosinophil-deficient mouse models and mAb studies in human subjects suggests that eosinophil depletion has a negligible effect on immunity to infection and other essential host functions.^{26,28,49} Moreover, IL-5 is classified as a lineage-specific cytokine for eosinophils only and has a central role in the differentiation, activation, and survival of eosinophils exclusively. Hence depletion of this cytokine is expected to have limited negative effects on other lineages than eosinophils. Therefore a treatment that specifically targets eosinophils seems to have limited effects on the host. Indeed, no safety issues were reported in any of the human trials or after-market authorization.^{49,50} In line with these findings, eIL-5-CMV_{TT} vaccine was well tolerated in horses and did not predispose to increased infections. Specifically, and given the fact that eosinophils are known to have antiparasitic effects and horses are natural hosts of certain intestinal helminths, no difference in helminth burden was found during the study period when comparing vaccinated and placebo-treated horses. However, more detailed safety studies will be required because of limitations of our placebo-controlled randomized clinical study, including the number of horses and the facts that we only included Icelandic horses and do not know yet the effect on helminth burden during long-term treatment.

Two injections of eIL-5-CMV_{TT} formulated in PBS induced high levels of anti-self antibodies recognizing eIL-5. Vaccine booster immunizations maintained these levels, whereas antibody responses decreased in the absence of further injections. Hence vaccination against IL-5 induced high levels of antibodies that were reversible in the absence of booster injections. High antibody titers were also measured against CMV_{TT}-VLPs and were used as a surrogate marker of vaccination because the immune system readily induces antibodies against the foreign CMV particle. Responses against IL-5 were slightly more variable than responses against the carrier VLPs, but responses against IL-5 and the CMV carrier correlated well overall. This is consistent with observations in human subjects immunized against IL-1 β and might indicate that the tightly packed VLP surface is able to activate the immune system more consistently than the IL-5 covalently attached to VLPs.⁵¹ Furthermore, and in contrast to anti-CMV antibodies, anti-IL-5 antibodies can bind to host IL-5 in the serum, and thus antibodies involved in

antigen-antibody complexes might no longer be detectable in the ELISA performed in this study. Natural variations in IL-5 levels between subjects will perhaps lead to different rates of depletion of anti-IL-5 antibodies and hence provide a perception of variability in anti-IL-5 antibody responses compared with CMV.

Similarly, as seen with anti-IL-5 mAbs in human subjects, we showed that vaccine-induced anti-IL-5 autoantibodies in horses were able to treat eosinophil-mediated symptoms. Clinical scores improved in eIL-5-CMV_{TT}-vaccinated horses when compared with the previous season of the same horses and placebo-treated horses of the same season. One major advantage of vaccines over mAbs is that there is no induction of antitherapeutic antibodies by the host because these polyclonal vaccine-induced antibodies are self-made. This ensures that the therapeutic vaccination can be given each year before the IBH season starts, leading to high antibody titers during the disease period and decreasing antibody titers during the unaffected time of year. For human subjects, antibodies induced against mAbs are a tangible problem that can limit the choice of therapeutics, an issue commonly seen in certain mAbs (eg, infliximab).^{52,53} Along these lines, to date, it is unclear how inbred the different horses are with respect to antibody allotypes. Therefore immunogenicity of the different horse breeds will be difficult to predict.

Another advantage of the vaccine's approach is the low cost of goods because much lower amounts of protein are needed for immunization compared with mAbs, which are usually applied based on body weight. Replacing the costly monoclonal therapies with active vaccination might also be an attractive treatment modality in human subjects, providing relief to health insurance systems. Therefore it would be interesting and also worthwhile to compare an anti-human IL-5 mAb versus human IL-5-VLP vaccines in human subjects.

Blood eosinophil levels during an IBH season increased typically in the spring and reached a peak in June. IBH lesion development followed eosinophil counts, with some delay. Overall, eosinophil counts in the blood of placebo-treated and unmanipulated horses correlated well with skin lesion scores, suggesting that in an unbiased system eosinophil levels in blood are a good indicator of eosinophil counts in skin and lesion severity. However, this correlation was lost in horses vaccinated against eIL-5, most likely because eosinophils do not efficiently enter tissues in the absence of IL-5. Of note, in the follow-up year of this study, during which slightly greater anti-IL-5 responses were induced, a statistically significant reduction of eosinophils in blood was observed (unpublished data). An alternative explanation for the absence of a correlation between blood eosinophil counts and antibody titers in the present study might be that the vaccine in the current vaccine preparation contained free non-coupled IL-5, whereas in the follow-up study this free IL-5 has been removed.

Taken together, eIL-5-CMV_{TT} successfully induced autoantibodies and mediated a statistically significant reduction in lesion scores in vaccinated horses when compared with placebo-treated horses and the pretreatment season. This is the first time that an anticytokine vaccine has shown clinical efficacy for the treatment of a disease in the target species and might facilitate the development of a similar vaccine targeting IL-5 in human subjects.

We thank all the horse owners who participated with their horses in our clinical studies. We thank Michael Fontaine and Hans-Uwe Simon for helpful discussion.

Clinical implications: Long-term treatment of horses affected by recurrent insect-bite hypersensitivity might be possible by using a vaccine targeting IL-5 and thus limiting eosinophil development and recruitment to the affected organ skin.

REFERENCES

- Baker KP, Quinn PJ. A report on clinical aspects and histopathology of sweet itch. *Equine Vet J* 1978;10:243-8.
- Braverman Y, Ungar-Waron H, Frith K, Adler H, Danieli Y, Baker KP, et al. Epidemiological and immunological studies of sweet itch in horses in Israel. *Vet Rec* 1983;112:521-4.
- Anderson GS, Belton P, Jahren E, Lange H, Kleider N. Immunotherapy trial for horses in British Columbia with *Culicoides* (Diptera: Ceratopogonidae) hypersensitivity. *J Med Entomol* 1996;33:458-66.
- Kurotaki T, Narayama K, Oyamada T, Yoshikawa H, Yoshikawa T. Immunopathological study on equine insect hypersensitivity ("kasen") in Japan. *J Comp Pathol* 1994;110:145-52.
- Anderson GS, Belton P, Kleider N. *Culicoides obsoletus* (Diptera: Ceratopogonidae) as a causal agent of *Culicoides* hypersensitivity (sweet itch) in British Columbia. *J Med Entomol* 1991;28:685-93.
- Quinn PJ, Baker KP, Morrow AN. Sweet itch: responses of clinically normal and affected horses to intradermal challenge with extracts of biting insects. *Equine Vet J* 1983;15:266-72.
- Fadok VA, Greiner EC. Equine insect hypersensitivity: skin test and biopsy results correlated with clinical data. *Equine Vet J* 1990;22:236-40.
- Greiner EC, Fadok VA, Rabin EB. Equine *Culicoides* hypersensitivity in Florida: biting midges aspirated from horses. *Med Vet Entomol* 1990;4:375-81.
- Schaffartzik A, Hamza E, Janda J, Cramer R, Marti E, Rhyner C. Equine insect bite hypersensitivity: what do we know? *Vet Immunol Immunopathol* 2012;147:113-26.
- van Grevenhof EM, Ducro B, Heuven HC, Bijma P. Identification of environmental factors affecting the prevalence of insect bite hypersensitivity in Shetland ponies and Friesian horses in The Netherlands. *Equine Vet J* 2007;39:69-73.
- Steinman A, Peer G, Klement E. Epidemiological study of *Culicoides* hypersensitivity in horses in Israel. *Vet Rec* 2003;152:748-51.
- Halldorsdottir S, Larsen HJ. An epidemiological study of summer eczema in Icelandic horses in Norway. *Equine Vet J* 1991;23:296-9.
- Bjornsdottir S, Sigvaldadottir J, Brostrom H, Langvad B, Sigurdsson A. Summer eczema in exported Icelandic horses: influence of environmental and genetic factors. *Acta Vet Scand* 2006;48:3.
- Brostrom H, Larsson A, Troedsson M. Allergic dermatitis (sweet itch) of Icelandic horses in Sweden: an epidemiological study. *Equine Vet J* 1987;19:229-36.
- Marti E, Gerber H, Lazary S. On the genetic basis of equine allergic diseases: II. Insect bite hypersensitivity. *Equine Vet J* 1992;24:113-7.
- Cunningham FM, Dunkel B. Equine recurrent airway obstruction and insect bite hypersensitivity: understanding the diseases and uncovering possible new therapeutic approaches. *Vet J* 2008;177:334-44.
- Hellberg W, Mellor PS, Torsteinsdottir S, Marti E. Insect bite hypersensitivity in the horse: comparison of IgE-binding proteins in salivary gland extracts from *Simulium vittatum* and *Culicoides nubeculosus*. *Vet Immunol Immunopathol* 2009;132:62-7.
- Kurotaki T, Narayama K, Arai Y, Arai S, Oyamada T, Yoshikawa H, et al. Langerhans cells within the follicular epithelium and the intradermal sweat duct in equine insect hypersensitivity "Kasen". *J Vet Med Sci* 2002;64:539-41.
- McKelvie J, Foster AP, Cunningham FM, Hamblin AS. Characterisation of lymphocyte subpopulations in the skin and circulation of horses with sweet itch (*Culicoides* hypersensitivity). *Equine Vet J* 1999;31:466-72.
- Pichler WJ. Delayed drug hypersensitivity reactions. *Ann Intern Med* 2003;139:683-93.
- Benarafa C, Collins ME, Hamblin AS, Cunningham FM. Role of the chemokine eotaxin in the pathogenesis of equine sweet itch. *Vet Rec* 2002;151:691-3.
- Riek RF. Studies on allergic dermatitis (Queensland itch) of the horse I. Description, distribution, symptoms and pathology. *Aust Vet J* 1953;29:177-84.
- Bandeira-Melo C, Woods LJ, Phoofolo M, Weller PF. Intracrine cysteinyl leukotriene receptor-mediated signaling of eosinophil vesicular transport-mediated interleukin-4 secretion. *J Exp Med* 2002;196:841-50.

24. Foster AP, McKelvie J, Cunningham FM. Inhibition of antigen-induced cutaneous responses of ponies with insect hypersensitivity by the histamine-1 receptor antagonist chlorpheniramine. *Vet Rec* 1998;143:189-93.
25. Strath M, Dent L, Sanderson C. Infection of IL5 transgenic mice with *Mesocricetus* induces very high levels of IL5 but depressed production of eosinophils. *Exp Hematol* 1992;20:229-34.
26. Kopf M, Brombacher F, Hodgkin PD, Ramsay AJ, Milbourne EA, Dai WJ, et al. IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity* 1996;4:15-24.
27. Collins PD, Marleau S, Griffiths-Johnson DA, Jose PJ, Williams TJ. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J Exp Med* 1995;182:1169-74.
28. Park YM, Bochner BS. Eosinophil survival and apoptosis in health and disease. *Allergy Asthma Immunol Res* 2010;2:87-101.
29. Zou Y, Sonderegger I, Lipowsky G, Jennings GT, Schmitz N, Landi M, et al. Combined vaccination against IL-5 and eotaxin blocks eosinophilia in mice. *Vaccine* 2010;28:3192-200.
30. Robinson DS. Mepolizumab for severe eosinophilic asthma. *Expert Rev Respir Med* 2013;7:13-7.
31. Leon-Ferre RA, Weiler CR, Halfdanarson TR. Hypereosinophilic syndrome presenting as an unusual triad of eosinophilia, severe thrombocytopenia, and diffuse arterial thromboses, with good response to mepolizumab. *Clin Adv Hematol Oncol* 2013;11:317-9.
32. Ortega HG, Yancey SW, Mayer B, Gunsoy NB, Keene ON, Bleecker ER, et al. Severe eosinophilic asthma treated with mepolizumab stratified by baseline eosinophil thresholds: a secondary analysis of the DREAM and MENSA studies. *Lancet Respir Med* 2016;4:549-56.
33. Pavord ID. Mepolizumab, quality of life, and severe eosinophilic asthma. *Lancet Respir Med* 2017;5:362-3.
34. Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A, et al. Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* 2009;360:973-84.
35. Smith DA, Minthorn EA, Beerah M. Pharmacokinetics and pharmacodynamics of mepolizumab, an anti-interleukin-5 monoclonal antibody. *Clin Pharmacokinet* 2011;50:215-27.
36. Chackerian B. Virus-like particles: flexible platforms for vaccine development. *Expert Rev Vaccines* 2007;6:381-90.
37. Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat Rev Immunol* 2010;10:787-96.
38. Pumpens P, Renhofa R, Dishlers A, Kozlovskaya T, Ose V, Pushko P, et al. The true story and advantages of RNA phage capsids as nanotools. *Intervirology* 2016;59:74-110.
39. Zeltins A. Construction and characterization of virus-like particles: a review. *Mol Biotechnol* 2013;53:92-107.
40. Zeltins A, West J, Zabel F, El Turabi A, Balke I, Haas S, et al. Incorporation of tetanus-epitope into virus-like particles achieves vaccine responses even in older recipients in models of psoriasis, Alzheimer's and cat allergy. *NPJ Vaccines* 2017;2:30.
41. Schmid F. Spectroscopic techniques to study protein folding and stability. In: Bucher J, Kiefhaber T (editors). *Protein folding handbook*. 2008. <https://doi.org/10.1002/9783527619498.ch2>.
42. Bachmann MF, Dyer MR. Therapeutic vaccination for chronic diseases: a new class of drugs in sight. *Nat Rev Drug Discov* 2004;3:81-8.
43. Milburn MV, Hassell AM, Lambert MH, Jordan SR, Proudfoot AE, Graber P, et al. A novel dimer configuration revealed by the crystal structure at 2.4 Å resolution of human interleukin-5. *Nature* 1993;363:172-6.
44. Jegerlehner A, Wiesel M, Dietmeier K, Zabel F, Gatto D, Saudan P, et al. Carrier induced epitopic suppression of antibody responses induced by virus-like particles is a dynamic phenomenon caused by carrier-specific antibodies. *Vaccine* 2010;28:5503-12.
45. Jonsdottir S, Svansson V, Stefansson SB, Schupbach G, Rhyner C, Marti E, et al. A preventive immunization approach against insect bite hypersensitivity: intralymphatic injection with recombinant allergens in Alum or alum and monophosphoryl lipid A. *Vet Immunol Immunopathol* 2016;172:14-20.
46. Walford HH, Doherty TA. Diagnosis and management of eosinophilic asthma: a US perspective. *J Asthma Allergy* 2014;7:53-65.
47. Czech W, Krutmann J, Schopf E, Kapp A. Serum eosinophil cationic protein (ECP) is a sensitive measure for disease activity in atopic dermatitis. *Br J Dermatol* 1992;126:351-5.
48. Oldhoff JM, Darsow U, Werfel T, Katzer K, Wulf A, Laifaoui J, et al. Anti-IL-5 recombinant humanized monoclonal antibody (mepolizumab) for the treatment of atopic dermatitis. *Allergy* 2005;60:693-6.
49. Legrand F, Klion AD. Biologic therapies targeting eosinophils: current status and future prospects. *J Allergy Clin Immunol Pract* 2015;3:167-74.
50. Mukherjee M, Sehmi R, Nair P. Anti-IL5 therapy for asthma and beyond. *World Allergy Organ J* 2014;7:32.
51. Cavelti-Weder C, Timper K, Seelig E, Keller C, Osranek M, Lassing U, et al. Development of an interleukin-1beta vaccine in patients with type 2 diabetes. *Mol Ther* 2016;24:1003-12.
52. Steenholdt C, Svenson M, Bendtzen K, Thomsen OO, Brynskov J, Ainsworth MA. Severe infusion reactions to infliximab: aetiology, immunogenicity and risk factors in patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2011;34:51-8.
53. Steenholdt C, Bendtzen K, Brynskov J, Thomsen OØ, Ainsworth MA. Measurement of infliximab and anti-infliximab antibody levels can help distinguish maintenance versus loss of response. *Gastroenterol Hepatol (N Y)* 2012;8:131-4.

METHODS

MALDI-MS/MS and HPLC of digested eIL-5–C-His

MALDI-MS/MS and HPLC of digested eIL-5–C-His were performed by the Functional Genomic Center, Zurich. eIL-5 was freshly digested with 1 μ g of trypsin (20:1) in 0.1% TFA/PBS buffer, pH 5 to 6, and analyzed by using 2

different HPLC columns (C8 and C18). Samples were mixed 1:1 with matrix solution (1.4 mg/mL alpha-4-hydroxycinnamic acid in 85% cerium nitrate, 0.1% trifluoroacetic acid, and 1 mmol/L MALDI-MS/MS and HPLC of digested eIL-5–C-His $\text{NH}_4\text{H}_2\text{PO}_4$) and spotted on the target. MALDI-MS/MS measurements were performed manually.

[SCORE SHEET LESION GRADING]										1/4
STUDY TITLE		„Therapeutic Vaccination against Equine Insect Bite Hypersensitivity (IBH) – Impfung gegen Sommerkezm“								
STUDY ID										
DATE, PLACE										
OWNER										
HORSE NAME / PATIENT ID										
Points are given for each summer eczema site. Every site is judged for intensity on location, size/diameter, blood, hairloss, scales, crust and lichenification. Per site intensities are graded from 1 to maximal 4 points. Total points is sum of all points. For more information see page 4.										
LOCATION										
		UP	MIDDLE	DOWN	LIGHT	STRONG	POINTS			
TAIL	☐	☐	☐	☐	☐	☐				
MANE	☐	☐	☐	☐	☐	☐				
BELLY	☐	☐	☐	☐	☐	☐				
WITHERS	☐	☐	☐	☐	☐	☐				
FLANK	☐	☐	☐	☐	☐	☐				
FACE	☐	☐	☐	☐	☐	☐				
EAR	☐	☐	☐	☐	☐	☐				
LEGS	☐	☐	☐	☐	☐	☐				
CROUP	☐	☐	☐	☐	☐	☐				
OTHER	☐	☐	☐	☐	☐	☐				
SIZE, DIAMETER										
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TAIL	< 0.5 cm ☐	0.5 ≤ x < 1 cm ☐	1 ≤ x < 2 cm ☐	≥ 2 cm ☐						
MANE	< 0.5 cm ☐	0.5 ≤ x < 1 cm ☐	1 ≤ x < 2 cm ☐	≥ 2 cm ☐						
BELLY	< 0.5 cm ☐	0.5 ≤ x < 1 cm ☐	1 ≤ x < 2 cm ☐	≥ 2 cm ☐						
WITHERS	< 0.5 cm ☐	0.5 ≤ x < 1 cm ☐	1 ≤ x < 2 cm ☐	≥ 2 cm ☐						
FLANK	< 0.5 cm ☐	0.5 ≤ x < 1 cm ☐	1 ≤ x < 2 cm ☐	≥ 2 cm ☐						
FACE	< 0.5 cm ☐	0.5 ≤ x < 1 cm ☐	1 ≤ x < 2 cm ☐	≥ 2 cm ☐						
EAR	< 0.5 cm ☐	0.5 ≤ x < 1 cm ☐	1 ≤ x < 2 cm ☐	≥ 2 cm ☐						
LEGS	< 0.5 cm ☐	0.5 ≤ x < 1 cm ☐	1 ≤ x < 2 cm ☐	≥ 2 cm ☐						
CROUP	< 0.5 cm ☐	0.5 ≤ x < 1 cm ☐	1 ≤ x < 2 cm ☐	≥ 2 cm ☐						
OTHER	< 0.5 cm ☐	0.5 ≤ x < 1 cm ☐	1 ≤ x < 2 cm ☐	≥ 2 cm ☐						
ANTONIA FETTELSCHOSS 2014, UPDATED 2015										

[SCORE SHEET LESION GRADING]										2/4
BLOOD										
									POINTS	
TAIL	INTACT EPIDERMIS ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
MANE	INTACT EPIDERMIS ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
BELLY	INTACT EPIDERMIS ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
WITHERS	INTACT EPIDERMIS ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
FLANK	INTACT EPIDERMIS ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
FACE	INTACT EPIDERMIS ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
EAR	INTACT EPIDERMIS ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
LEGS	INTACT EPIDERMIS ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
CROUP	INTACT EPIDERMIS ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
OTHER	INTACT EPIDERMIS ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
HAIRLOSS										
									POINTS	
TAIL	MILD ☐	MODERATE ☐	SEVERE ☐	NO HAIR ☐						
MANE	MILD ☐	MODERATE ☐	SEVERE ☐	NO HAIR ☐						
BELLY	MILD ☐	MODERATE ☐	SEVERE ☐	NO HAIR ☐						
WITHERS	MILD ☐	MODERATE ☐	SEVERE ☐	NO HAIR ☐						
FLANK	MILD ☐	MODERATE ☐	SEVERE ☐	NO HAIR ☐						
FACE	MILD ☐	MODERATE ☐	SEVERE ☐	NO HAIR ☐						
EAR	MILD ☐	MODERATE ☐	SEVERE ☐	NO HAIR ☐						
LEGS	MILD ☐	MODERATE ☐	SEVERE ☐	NO HAIR ☐						
CROUP	MILD ☐	MODERATE ☐	SEVERE ☐	NO HAIR ☐						
OTHER	MILD ☐	MODERATE ☐	SEVERE ☐	NO HAIR ☐						
SCALES										
									POINTS	
TAIL	NONE ☐	TINY, FEW ☐	MODERATE, MID-SIZE ☐	MANY, BIG ☐						
MANE	NONE ☐	TINY, FEW ☐	MODERATE, MID-SIZE ☐	MANY, BIG ☐						
BELLY	NONE ☐	TINY, FEW ☐	MODERATE, MID-SIZE ☐	MANY, BIG ☐						
WITHERS	NONE ☐	TINY, FEW ☐	MODERATE, MID-SIZE ☐	MANY, BIG ☐						
FLANK	NONE ☐	TINY, FEW ☐	MODERATE, MID-SIZE ☐	MANY, BIG ☐						
FACE	NONE ☐	TINY, FEW ☐	MODERATE, MID-SIZE ☐	MANY, BIG ☐						
EAR	NONE ☐	TINY, FEW ☐	MODERATE, MID-SIZE ☐	MANY, BIG ☐						
LEGS	NONE ☐	TINY, FEW ☐	MODERATE, MID-SIZE ☐	MANY, BIG ☐						
CROUP	NONE ☐	TINY, FEW ☐	MODERATE, MID-SIZE ☐	MANY, BIG ☐						
OTHER	NONE ☐	TINY, FEW ☐	MODERATE, MID-SIZE ☐	MANY, BIG ☐						
ANTONIA FETTELSCHOSS 2014, UPDATED 2015										

[SCORE SHEET LESION GRADING]										3/4
CRUST										
									POINTS	
TAIL	NONE ☐	TINY ☐	HALF ☐	TOTAL ☐						
MANE	NONE ☐	TINY ☐	HALF ☐	TOTAL ☐						
BELLY	NONE ☐	TINY ☐	HALF ☐	TOTAL ☐						
WITHERS	NONE ☐	TINY ☐	HALF ☐	TOTAL ☐						
FLANK	NONE ☐	TINY ☐	HALF ☐	TOTAL ☐						
FACE	NONE ☐	TINY ☐	HALF ☐	TOTAL ☐						
EAR	NONE ☐	TINY ☐	HALF ☐	TOTAL ☐						
LEGS	NONE ☐	TINY ☐	HALF ☐	TOTAL ☐						
CROUP	NONE ☐	TINY ☐	HALF ☐	TOTAL ☐						
OTHER	NONE ☐	TINY ☐	HALF ☐	TOTAL ☐						
LICHENIFICATION / SWELLING										
									POINTS	
TAIL	NONE ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
MANE	NONE ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
BELLY	NONE ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
WITHERS	NONE ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
FLANK	NONE ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
FACE	NONE ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
EAR	NONE ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
LEGS	NONE ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
CROUP	NONE ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
OTHER	NONE ☐	MILD ☐	MODERATE ☐	SEVERE ☐						
SHEATH / UDDER										
									POINTS	
SHEATH	GRADE 1 ☐	GRADE 2 ☐	GRADE 3 ☐	GRADE 4 ☐						
UDDER	GRADE 1 ☐	GRADE 2 ☐	GRADE 3 ☐	GRADE 4 ☐						
TOTAL POINTS										
NOTES										
VETERINARY RESEARCHER										
ANTONIA FETTELSCHOSS 2014, UPDATED 2015										

[SCORE SHEET LESION GRADING]										4/4
LESION SCORING										
For IBH symptom scoring, the location (tail, mane, belly, flank, face, ear, leg, and the like) where IBH lesions occur are recorded. Each location is divided into 3 parts: up, middle, down. Further according to the number of lesions, each location is classified into light and strong. Dependent on how many parts are affected (up / middle / down) and how many lesions per location are found (light / strong), 1 to 4 points can be scored (1 point = one part affected, lesion light; 4 points = all three parts affected, lesion strong). Moreover, these locations are classified for 6 further properties: size (diameter), blood, hair loss, scales, crust, and lichenification / swelling. For all these properties also 1 to 4 points can be scored. Size is divided into < 0.5 cm (1 point), 0.5 ≤ x < 1 cm (2 points), 1 ≤ x < 2 cm (3 points), and ≥ 2 cm (4 points). Blood is divided into intact epidermis (1 point), mild (2 points), moderate (3 points), and severe (4 points). Hair loss is divided into mild (1 point), moderate (2 points), severe (3 points), and no hair (4 points). Scales is divided into none (1 point), tiny, few (2 points), moderate, mid-size (3 points), and many, big (4 points). Crust is divided into none (1 point), tiny (2 points), half (3 points), and total (4 points). Lichenification and/or swelling is divided into none (1 point), mild (2 points), moderate (3 points), and severe (4 points). Additionally, if sheath or udder is swollen, minimally 5 or maximally 20 points can be scored: grade 1 (5 points), grade 2 (10 points), grade 3 (15 points), and grade 4 (20 points). Finally all points are added up and are the IBH symptom score.										

FIG E1. Score sheet lesion scoring. Results are as described in the [Methods](#) section of the main article.

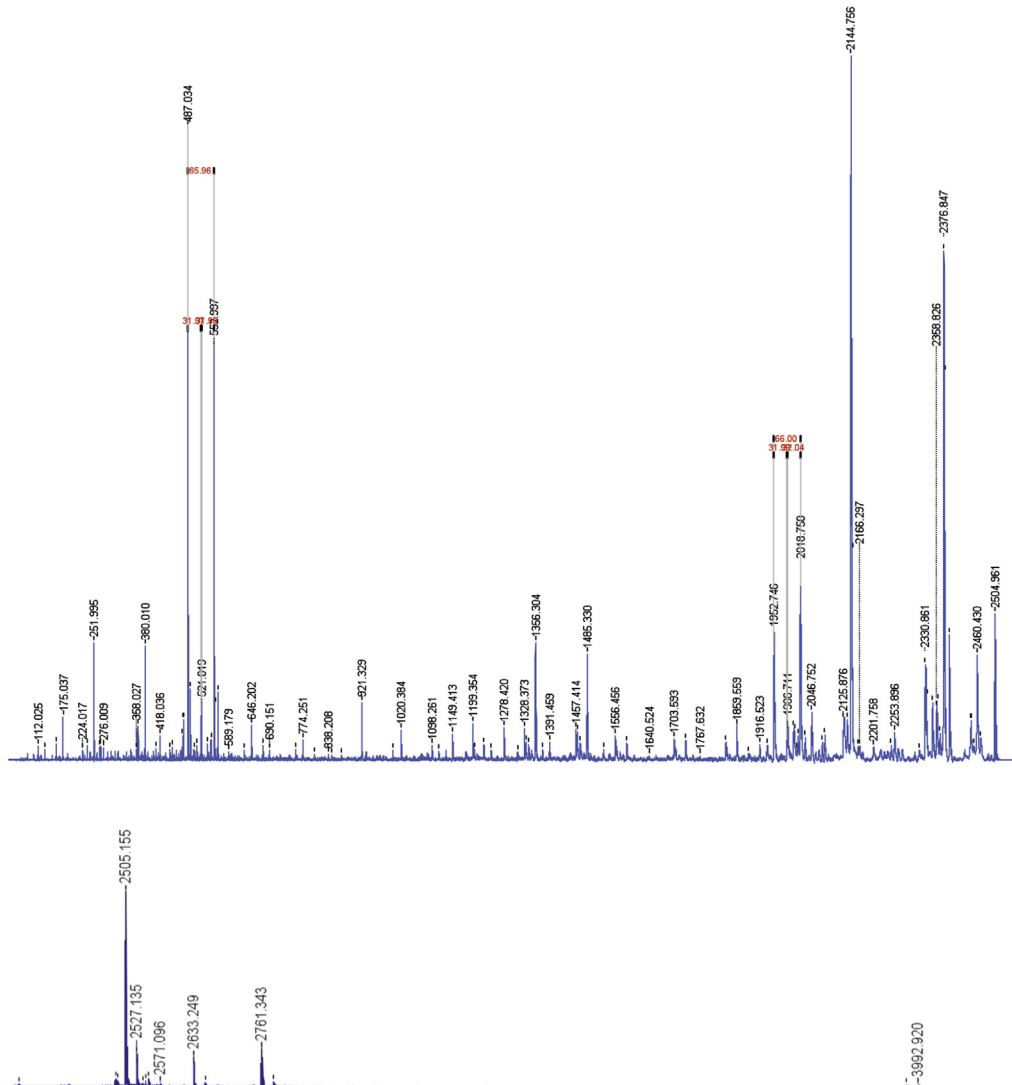


FIG E2. Liquid chromatography–MALDI-MS/MS of purified and trypsin-digested eIL-5–C–His. Dimers were linked intermolecularly through 2 disulfide bridges through Cys44 of one monomer and Cys86 of the other and *vice versa*.

Señor Director

NUEVAS CREACIONES

Superintendencia de Industria y Comercio

E. S. D.

Referencia: Expediente NC2017/0004119

Asunto: Solicitud de patente para PARTÍCULAS TIPO VIRUS MODIFICADAS DE CMV

Solicitante: SAIBA GMBH

Fecha de solicitud: 27 de abril de 2017

RESPUESTA A SEGUNDO EXAMEN DE PATENTABILIDAD

JOSE ANDRES RINCON USATEGUI, identificado como aparece al pie de mi firma, en mi condición de apoderado de la sociedad solicitante de la patente de la referencia, doy respuesta al Oficio **12157**, notificado por fijación en lista el 09 de octubre de 2018.

I. MODIFICACIONES AL CAPÍTULO REIVINDICATORIO

En respuesta al examen de patentabilidad aportó un Capítulo Reivindicatorio modificado conformado por **54** reivindicaciones en las que se han incluido las siguientes modificaciones:

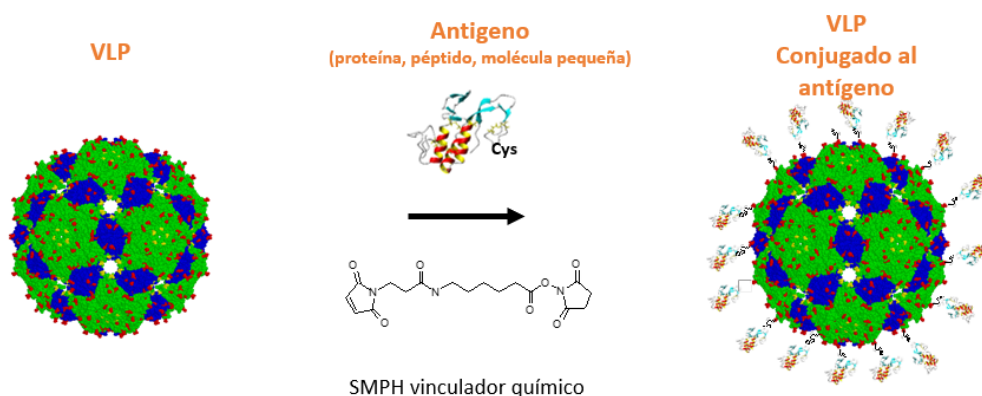
1. Se han eliminado las reivindicaciones 2 a 5, 30, 33, 38, 51 y 58;
2. En la reivindicación 1 se ha remplazado la expresión *“por lo menos un”* por *“una pluralidad”*;
3. En el numeral *b)* de la reivindicación 1 se ha especificado las posibles secuencias del epítipo de célula T ayudante;
4. En la nueva reivindicación 5 (antigua reivindicación 9) se ha remplazado la expresión *“al menos”* por *“uno o más”* con el fin de dar claridad sobre la materia reclamada;

5. En la nueva reivindicación 15 (antigua reivindicación 19) se ha remplazado la expresión “*por lo menos*” por “*una o más*”;
6. En la nueva reivindicación 22 (antigua reivindicación 26) se han eliminado las expresiones “*al menos*” y “*máximo*”;
7. Se han ajustado las dependencias de las reivindicaciones de acuerdo con las modificaciones realizadas.

Estas modificaciones se ajustan al Artículo 34 de la Decisión 486 de 2000, ya que no constituyen una ampliación de la protección que correspondería a la divulgación contenida en la solicitud inicial.

II. EN CUANTO A LA SUPUESTA FALTA DE CLARIDAD

1. El Examinador ha considerado que el capítulo reivindicatorio no era conciso porque se reclamaba la misma materia en más de una reivindicación. Con respecto a la objeción del Examinador es preciso resaltar que, tal y como se señaló en el memorial 1 del presente memorial se han eliminado las antiguas reivindicaciones 30, 33, 37, 51 y 58 que reclamaban de manera mas especifica la materia de las antiguas reivindicaciones 29, 32, 37 y 51. De esta manera se da por superada esta objeción.
2. Con respecto a la objeción del Examinador según la cual la reivindicación 1 no era concisa por comprender la expresión “*por lo menos un polipéptido de CMV modificado*”, se desea respetuosamente aclarar que la partícula tipo virus reivindicada está compuesta típicamente por una pluralidad de polipéptidos modificados de CMV (aprox. 180; pág. 3, líneas 9 a 10), los cuales están auto ensamblados para formar la partícula tal y como se reivindica. Para mayor ilustración, a continuación, se muestra una representación gráfica de la partícula tipo virus reivindicada:



De manera similar, y como se observa en la representación, los antígenos no están insertados en la secuencia del polipéptido de CMV, sino que estos están acoplados químicamente.

Así las cosas, restringir el número de polipéptidos CMV sería una limitación excesiva del alcance del concepto inventivo desarrollado por el Solicitante. Sin embargo, con el único fin de darle claridad a la reivindicación 1 se ha remplazado el fragmento objetado por *“una pluralidad”*.

Finalmente, con el fin de dar aún mayor claridad sobre la materia reclamada se presentar anexos a este memorial dos artículos científicos publicados por los inventores (Fettelschoss *et al.*, 2018 y Zeltins *et al.*, 2017) en los cuales se han subrayado los fragmentos relacionados con la con figuración de la partícula tipo virus.

Teniendo en cuenta la modificación y las aclaraciones realizadas, se debe dar por superada esta objeción.

3. De manera similar, el Examinador ha objetado la reivindicación 1 por no especificar las secuencias de las secuencias de aminoácidos de los epítopes PADRE o toxina tetánica en su componente. Al respecto es pertinente señalar una vez más que en la nueva reivindicación 1 se han especificado las posibles secuencias de los aminoácidos del componente **b)** de la partícula tipo virus modificada.

Así las cosas, la nueva reivindicación 1 enmendada es concisa y se dan por superadas las objeciones del Examinador.

4. El Examinador ha afirmado que las antiguas reivindicaciones 1, 9, 15, 19, 26, 41 y 42 no eran concisas por comprender expresiones como *“por lo menos”, “máximo 20 aminoácidos”, “al menos”, “que consiste en 6 a 14 aminoácidos” “derivado de (...)”*, que se consideran imprecisas. Con respecto a lo anterior se hace necesario poner de presente lo siguiente:

- En relación con la reivindicación 1 se desea respetuosamente llamar la atención del Examinador sobre el hecho de que la partícula VLP modificada que se reclama en la presente solicitud está compuesta por una pluralidad (típicamente 180 copias) del polipéptido CMV modificado, el cual se auto ensambla para formar una VLP; En consecuencia, definir el número de antígenos unidos sería una limitación excesiva de

alcance de la invención reivindicada; sin embargo como se mencionó anteriormente se han realizado modificaciones con fines aclaratorios.

- De manera similar, y con respecto a las expresiones “*por lo menos*” y “*al menos*” (antiguas reivindicaciones 9, 15, 19, 26), se desea respetuosamente señalar nuestro desacuerdo, en el sentido que esta expresión es perfectamente clara ya que establece los requisitos mínimos para que una persona medianamente versada en la materia pueda reproducir la invención, teniendo en cuenta que la limitación de la materia a un rango o valor específico constituiría una limitación excesiva al alcance que dejaría por fuera múltiples posibles configuraciones que alcanzarían de igual manera el efecto deseado y solucionarían el problema técnico que enfrenta la invención. Sin embargo, con el único fin de agilizar el trámite el Solicitante ha remplazado la expresión objetada por el término “*uno o más*”.
- Por otra parte, la expresión “*máximo*” (antigua reivindicación 22) ha sido eliminada del capítulo reivindicatorio modificado.
- Con respecto a las expresiones “*un péptido derivado de α -sinucleína*” y “*que consiste en 6 a 14 aminoácidos*” se desea respetuosamente manifestar nuestro desacuerdo con el Examinador, en el sentido que las expresiones objetadas son perfectamente claras para cualquier persona medianamente versada en la materia. Adicionalmente, las mismas se encuentran debidamente soportadas en la descripción originalmente presentada.

Teniendo en cuenta las aclaraciones y modificaciones realizadas se entiende como superada cualquier objeción relacionada con las expresiones mencionadas por el Examinador.

5. El Examinador afirma que la antigua reivindicación 9 (nueva reivindicación 5) no era clara por referirse a una composición que comprendía un único componente (una partícula tipo virus modificada). Con respecto a esta objeción se desea respetuosamente llamar la atención del Examinador sobre el hecho de que la composición reclamada no está conformada por un único componente, sino más bien está compuesta por un VLP modificado y una pluralidad de antígenos. Teniendo en cuenta lo anterior debe considerarse como superada la objeción formulada en relación con la nueva reivindicación 5.

6. El Examinador ha objetado las antiguas reivindicaciones 9 a 27 (nuevas reivindicaciones 5 a 23) porque supuestamente no se encontraban soportadas en la descripción por comprender expresiones como: “*comprende al menos un primer sitio de unión y (b) al menos un antígeno (...) al menos un segundo sitio de unión (...)*”. Al respecto

se desea respetuosamente llamar la atención del Examinador sobre el objeto de la invención, en donde se ACOPLA químicamente un gran número de ANTIGENOS y se fusiona solo un epítipo de célula T ayudante dentro de la cápside. En tal sentido, especificar un número o rango exacto dejaría una vez más por fuera una gran posibilidad de configuraciones de la partícula tipo virus reivindicada. Si embargo, con el único fin de darle mayor claridad a las reivindicaciones, se ha reemplazado la expresión “*al menos*” por “*uno o más*”.

Teniendo en cuenta las aclaraciones y modificaciones realizadas, se da por superada esta objeción.

7. Con respecto a la objeción del Examinador según la cual las antiguas reivindicaciones 9 a 16 (nuevas reivindicaciones 5 a 12) porque supuestamente en la descripción no se evidencia ningún ejemplo de VLP vinculada a epítopes de manera no covalente es preciso una vez más llamar la atención del Examinador sobre la materia reclamada en la Solicitud. Mas específicamente se desea recordar que los antígenos NO están insertados en la secuencia de CMV, sino que están acoplados químicamente a la partícula tipo virus por un enlace peptídico no-covalente, tal y como se mostró gráficamente (*numeral II.2* del presente memorial). Sólo el epítipo de la célula T ayudante está insertado dentro de las secuencias de CMV. Teniendo en cuenta lo anterior, esta objeción debe darse como superada.

8. El Examinador ha objetado las antiguas reivindicaciones 19 a 27, 34, 39, 40 a 42, 46, 47, 52, 59, 60, 62 y 63 afirmando *que no se evidencian composiciones con antígenos tumorales, así como tampoco se evidencian composiciones con agentes inmunoestimulantes de ácidos nucleicos*.

Respecto a lo anterior se desea llamar la atención del Examinador sobre las líneas 25 de la página 98 a la línea 23 de la página 99, de la descripción originalmente presentada, en donde está claramente soportado y especificado el uso de composiciones con antígenos tumorales. De manera similar, el uso de sustancias inmunoestimulantes de ácidos nucleicos está soportado en la descripción en las líneas 7 de la página 58 a la línea 21 de la página 59.

Así mismo, deseamos se desea indicar que dentro de una solicitud de patente no es factible ni posible presentar resultados experimentales para todas y cada una de las modalidades preferidas descritas en la descripción. Así las cosas, la materia reclamada en las reivindicaciones es una generalización razonable de las realizaciones enseñadas en la descripción y por tanto se debe dar por superada esta objeción.

9. Finalmente, el Examinador ha dicho que las antiguas reivindicaciones 62 y 63 (nuevas reivindicaciones 53 y 54) no eran concisas por reclamar una composición o una vacuna en una misma reivindicación. Respecto a lo anterior se desea manifestar nuestro desacuerdo, ya que ambas reivindicaciones reclaman de manera independiente una vacuna y una composición farmacéutica. Así las cosas, se le solicita amablemente al Examinador retirar la objeción planteada a este respecto.

Con base en todo lo anterior, considero que el Capítulo Reivindicatorio modificado adjunto cumple con todos los requisitos de claridad y concisión, ajustándose así a los lineamientos del Artículo 30 de la Decisión 486, ya que cualquier persona del oficio normalmente versada en la materia está en completa capacidad de reproducir la invención sin ambigüedad alguna.

III. EN CUANTO A LA SUPUESTA FALTA DE SOPORTE EN LA DESCRIPCIÓN

El Examinador sostiene que la descripción no divulga la invención de manera suficientemente completa para que persona normalmente versada en la materia técnica correspondiente pueda ejecutarla por lo que no existe soporte para el desarrollo de partículas tipo virus (VLP) que comprendan cualquier antígeno, ni más de dos antígenos insertados en el péptido CP. Respecto a la objeción anterior, se desea una vez más recordar que el Solicitante ha incluido las secuencias de los componentes a) y b) de la partícula tipo virus modificada. Teniendo en cuenta lo anterior se ha limitado el alcance de la solicitud a una generalización razonable de la materia enseñada en la descripción y por tanto esta objeción debe darse por superada.

IV. EN CUANTO A LA SUPUESTA FALTA DE UNIDAD DE INVENCION

El Examinador ha afirmado que la Solicitud no cumple con el requisito de unidad de invención porque la partícula tipo virus (VLP) modificada del virus del mosaico del pepino (CMV) que comprende *(a) un polipéptido que consiste en una secuencia de una proteína de recubrimiento CMV y (b) un epítipo de célula T ayudante; no comparte una estructura esencial para la actividad común.* Adicionalmente, que el concepto inventivo común ya había sido enseñado por *Natilla A., et al., 2006.*

Respecto a lo anterior es preciso señalar que la partícula tipo virus enseñada por la técnica anterior (*Natilla et al., 2006*) no comprendía polipéptidos CMV en donde el epítipo de la célula T ayudante se fusiona a la N-terminal de dicho polipéptido CMV, y en donde dicha

región N-terminal de dicho polipéptido CMV corresponde a los aminoácidos 2 -12 de la SEC ID NO: 1. Adicionalmente Natilla *et al.*, no divulga ni sugiere que los epítomos de la célula T ayudadora sean de las secuencias definidas en la Opción 1 u Opción 2.

Teniendo en cuenta lo anterior, la presente solicitud no sólo cumple con el criterio de unidad de invención, sino que además es nueva e inventiva respecto al arte previo y especialmente frente al documento citado como *Natilla et al.* En consecuencia, se solicita amablemente proceder al otorgamiento de la solicitud de patente.

V. ESTADO DE LA TÉCNICA

A este respecto, se pone de presente que, de acuerdo con el examen de patentabilidad realizado por el examinador, no existe en el arte previo ninguna divulgación que anticipe las características de la presente invención. En otras palabras, la invención cumple a cabalidad los requisitos de aplicación industrial, novedad y nivel inventivo.

VI. PETICIÓN

1. Puesto que se ha dado respuesta a todas las objeciones formuladas por la Dirección de Nuevas Creaciones, solicito proceder al otorgamiento de la solicitud de patente.

2. Subsidiariamente, en caso de que su Despacho considere que la modificación introducida al Capítulo Reivindicatorio amerita revisión adicional, solicito un segundo examen de patentabilidad de conformidad con lo dispuesto en el Artículo 2 de la Resolución 3719 de 02 de febrero de 2016, para lo cual realizo el pago de tasa correspondiente.

VII. ENTREVISTA CON EXAMINADOR

Además de este memorial de respuesta he solicitado una entrevista en las oficinas de la Dirección de Nuevas Creaciones con el fin de discutir con el Examinador temas que considero de importancia para el trámite. Atentamente solicito no se resuelva este expediente hasta después de llevar a cabo dicha entrevista.

VIII. ANEXOS

1. Capítulo Reivindicatorio modificado conformado por 54 reivindicaciones;
2. Pago en línea por concepto de examen de patentabilidad adicional;
3. Artículos Fettelschoss *et al.*, 2018 y Zeltins *et al.*, 2017

Señor Director,

ANDRÉS RINCÓN USCÁTEGUI

C.C. 79.780.910 de Bogotá

T.P. 114.908