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Production and characterization of hypoallergenic virus-like nanoparticles shielding full-length mutated Art v 1 with membrane-bound murine IL-15 for immunomodulation of allergen-specific T cells

Bachelor thesis
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1. Introduction

1.1 Immunological basics

The immune system is divided into innate and adaptive immunity (Johansson et al. 2001). The innate immune system consists of different cellular and humoral factors such as macrophages, neutrophils, basophils, eosinophils, and the complement system. It also represents the host's first line of defence against microbes and is antigen-less-specific. The adaptive immunity on the other hand is mediated by T and B cells with antigen-specific receptors, T-cell receptors (TCR) and membrane-bound immunoglobulins (Ig) as B-cell antigen receptors. Adaptive immunity can also build an immunological memory in the form of memory B and T cells, against different target antigens for an immediate response upon repeated contact with the specific antigen (Averbeck et al. 2007, Chaplin 2010).

B cells are activated through the recognition of antigens by their cell-surface expressed Ig receptors. Activated B cells produce soluble immunoglobulins, which are divided into 5 classes according to their heavy chain: IgA, IgD, IgE, IgG and IgM.

IgM is expressed primarily on B cells during their maturation and has low affinity. The low affinity is compensated by multimeric interactions. The functions of IgM are opsonizing/tagging antigens for destruction and activating the complement system. IgG is the most frequent antibody in the blood and fulfils the same functions as IgM but with higher affinity and has a longer serum half-life. IgA is located on mucosal surfaces and neutralizes toxins, viruses, and bacteria directly, preventing binding to the mucosal surface and further colonization. IgE interacts with high-affinity IgE-receptor (FcεRI) on mast cells, basophils, Langerhans cells and eosinophils. They are closely related to hypersensitivity and allergic reactions as well as parasitic worm infections (Schroeder and Cavacini 2010). IgD is mainly expressed in a membrane-bound form together with IgM on the surface of B cells and in the context of allergy, it has been found recently, that IgD is also involved in binding and activating mast cells and basophils (Chen et al. 2009, Zhai et al. 2018). T-cell-stimulated B cells can perform immunoglobulin class switch recombination, which leads to th-e production of immunoglobulins other than IgM. For example, a switch towards IgE occurs when B cells are stimulated in the presence of secreted IL-4 and IL-13 by Thelper 2 cells (Averbeck et al. 2007, Chaplin 2010).

Naïve T cells are activated through peptide antigens, which are processed and displayed on class I or II major histocompatibility complex (MHC). $CD8^+$ naïve T cells recognize antigens on MHC class I molecules and differentiate into cytotoxic $CD8^+$ effector T cells and naïve CD4+ T cells recognize antigens on MHC class II. MHC class II is exclusively expressed on professional antigen presenting cells (APCs), i.e., dendritic cells (DC), monocytes, macrophages and B cells. Those APCs take up foreign antigens and present them via MHC class II molecules to naïve $CD4^+$ T-helper cells. Subsequently, $CD4^+$ T cells can differentiate into T-helper 1 (Th1 cells), T-helper 2 (Th2 cells), T-helper 17 (Th17 cells) or regulatory T cells (Treg cells), depending on the secreted cytokine profiles in the microenvironment and the surface-expressed molecules of APCs. For instance, interleukin (IL)-12 induces differentiation of $CD4^+$ T cells to Th1 cells, which further activate macrophages and cellular immunity and induce the production of opsonizing antibodies of the IgG subclass. On a different note, IL-4 and IL-13 induce differentiation to Th2 cells, which leads to the production and differentiation of B cells and antibody isotypes, including IgE, therefore humoral-mediated immunity. IL-6 activated Th17 cells further secrete IL-17, IL-17F, IL-21 and IL-22, leading to the recruitment of neutrophils and macrophages. Treg cells can be divided into many subpopulations and suppress other T cells, like Th1 and Th2 cells, following the regulation of allergic and non-allergic reactions. In addition, Treg cells can inhibit APCs, mast cells and the production of IgE (Gause 1999, Shevach 2006, Dong 2008).

1.2 Immunological principles of a Type I hypersensitivity reaction

Allergies are caused by hypersensitivity reactions of the immune system to harmless antigens. An allergic reaction starts with the sensitization of the immune system to the respective allergen. In this phase, the antigen is recognized via the MHC class II molecules on APCs leading to the differentiation of naïve $CD4^+$ T cells into Th2 cells in the presence of the cytokines IL-4 and IL-13. Subsequently, the Th2 cells start to secrete IL-4, IL-5, and L-13 and express the surface molecule CD40 ligand, which interacts with the CD40 molecule on B cells, all together stimulating the production and the class switch recombination towards IgE production in B cells. The soluble IgE binds to the FcεRI on mast cells and basophils. If an allergen encounters such IgE, cross-linking of the receptor via binding of IgE to the allergen occurs, leading to the activation and release of mediator molecules. Such molecules are vasoactive amines, lipid mediators, chemokines, and other cytokines, and lead to local reactions like constriction of airways, erythema or tissue swelling in the skin, edema or itching and are characteristic of the immediate phase of the allergic reaction. Systemic reactions

comprise urticaria, angioedema or even anaphylactic reactions (vascular collapse) in severe allergic cases. In the late phase of allergic reactions, the IgE bound to the FcεRI of dendritic cells and monocytes, as well as to the low-affinity IgE-receptors (FcεRII) on B cells, cause APCs to take up increased amounts of allergen and presenting it to the specific $CD4^+$ T cells. Late phase reactions appear two or more hours after the allergen exposure followed by a peak at 6–9 hours and resolve after 24–48 hours. Reactions include edema formation, pain, warmth, and erythema in the affected tissue and airway narrowing as well as mucus hypersecretion in the lungs (Broide 2001, Larché et al. 2006, Averbeck et al. 2007, Galli et al. 2008).

1.3 The major mugwort pollen allergen of Artemisia vulgaris

In western societies, more than 25 % of individuals are affected by allergies, including allergies to drugs, food, pets, latex, insects, mold and pollen (Kratzer et al. 2019). Among pollen allergies, one of the main causes in late summer and autumn are pollen deriving from *Artemesia vulgaris* (mugwort), with 10-14 % of all individuals suffering from pollinosis being sensitized to it. Pollen of the mugwort weed plant consists of at least nine allergenic proteins (Himly et al. 2002). The mugwort pollen allergen Art v 1 interacts with over 95 % of IgE from people allergic to mugwort and has therefore been entitled major mugwort pollen allergen (Jahn-Schmid et al. 2002). This allergen shows a head and tail structure with an N-terminal cysteine-rich domain, comparable to plant defensins, and a C-terminal proline-rich region with carbohydrate-based post-translational modifications. Both the defensin-fold and post-translational modifications are engaged in the recognition of the allergen by IgE antibodies, whereas the post-translational modifications do not contribute to the recognition by T cells, making recombinant non-post-translational modified Art v 1 a potential option for hypoallergenic vaccines (Himly et al. 2002). Experiments of Jahn-Schmid et al. revealed that all patients suffering from mugwort pollen allergies express the $CD4^+CD8^-$ αβTCR Th phenotype with most of them secreting Th2 cytokine profiles in reaction to interaction with Art v 1, causing allergic reactions. About 80 % of these $CD4^+CD8^-$ αβTCR-expressing T-cell clones recognize the immunodominant Art v 1 T-cell epitope, Art v $1_{22\text{-}36}$, which is presented to them by human-leukocyte antigens (HLA), corresponding to the class II MHC in humans. Inhibition tests showed that the presentation is restricted to the HLA-DR1 allele. The HLA-alleles are germ-line defined in all individuals, making the ones expressing HLA-DR1 susceptible to developing allergies to the mugwort major pollen antigen Art v 1. Notably, the genetic predisposition can be determined before sensitization with Art v 1 and therefore be prevented by preventive treatment, e.g., by novel remidies such as hypoallergenic VNP-based

vaccines targeting the immunodominant T-cell epitope and shifting the immune response in susceptible individuals to a Th1 prone cytokine profile in response to further contact with the allergen (Jahn-Schmid et al. 2002, Holdsworth et al. 2009).

1.4 Allergen-specific Immunotherapy

Current treatments for allergies are allergen-avoidance or various medications for reduction of the symptoms. Such medications for instance include antihistamines, decongestants, diverse asthma medications, or epinephrine (adrenaline) shots for acute reactions after allergen contact, for example, food or insect allergies. These treatments aim to make the allergy endurable due to temporary operating medications, but these are not lasting very long and further also cannot cure patients from suffering of allergic reactions in the future (American Academy of Allergy 2022). In this context allergen-specific immunotherapy (AIT) is being investigated. The fundamental principle of AIT is about a repeated intake of the allergen or derivates thereof with increasing doses of the allergen to achieve active immune tolerance and remains the only disease-modifying treatment for allergic reactions. The goal is to reduce the frequency and the severity of IgE-mediated reactions with a persistant effect after the treatment, rather than just avoiding the allergen or palliating the symptoms (Tephen et al. 1999a, 1999b, Larché et al. 2006, Maggi 2010). The first attempt to cure allergy to grass pollen by active immunization was performed in 1911 by administrating subcutaneous injections of grass pollen extract and has been improved since then (Bostock and Blackley 1911). Over the years, development of AIT focussed on two major issues, decreasing the side effects by for example linking the allergen to adjuvants for a slower release and reducing the allergenicity of the given allergen by modifying it and/or cutting the protein into smaller peptides (Larché et al. 2006). Nowadays, there are different targeting mechanisms when performing AIT. AIT can modify the responses of APCs, T cells and B cells in both, their quantity and function. Another aspect is a seasonal decrease of eosinophils and basophils in the mucosa as well as the IgE-induced release of histamine from basophils, and mast cells in the skin (Durham et al. 1999, Wilson et al. 2001, Shim et al. 2003).

One study in 2010 (Maggi 2010) proved that an administration of the allergen over a longer periods of time can induce and recruit allergen-specific Th1-like Treg 1 cells, followed by an Interferon (IFN)-γ-provoked activation of macrophages and dendritic cells (DC), like APCs as well as direct suppression of IgE-producing cells. In addition, the microenvironmental IL-12, IL-10 and transforming growth factor (TGF)-β were increased. IL-12 leads to the trans-differentiation of Th2 and Th17 cells into Th1 cells. IL-10 suppresses Th2 and Th17 cell

responses, inhibits mast cells, basophils and eosinophils and induces IgG4-production. TGF-β blocks Th2 cells and induces an IgA switch and the reduction of the activation of mast cells and eosinophils. Moreover, IFN-γ leads to an IgG1 switch and the direct suppression of IgE production. IgG1, IgG4 and IgA are allergen-specific blocking-antibodies, inhibiting IgE-mediated degranulation. However, the conditions in which DCs modify their microenvironment, leading to all these changes are not fully determined and still need to be established for further use in treatments. One approach to performing AIT is peptide immunotherapy with wild-type or altered peptide ligands (APL). Peptide immunotherapy focuses on T-cell epitopes of major allergens, like Art v 1, which are too small of being recognized by the IgE of the cell leading to reduced risk of type I immune reactions after their application (Candia et al. 2016).

1.5 Altered peptide ligands

Altered peptide ligands are immunogenic antigen-derived peptides with single amino-acid substitutions, which can stimulate different T-cell functions and subsequently modify the direction of immune responses (Joanne Sloan-Lancaster 1996).

In the context of AIT, peptide (APL) immunotherapy has in some studies been successfully applied for the therapy of patients suffering from multiple sclerosis and experimental autoimmune uveitis. T cells were primed with suitable APLs, leading to a desirable Th2 phenotype shift and the induction of Treg cells (Cortes et al. 2008, Kammona and Kiparissides 2020). APLs in treatments for allergic diseases are modified to stimulate Th1 and subsequent Interferon-production, leading to the activation of macrophages, followed by the induction of opsonizing IgG and cellular immunity (G. Tau 1999, Shevach 2006). To achieve these effects regarding mugwort allergies, Art v 1 APLs with single amino acid substitutions located in between the 25^{th} and 36^{th} amino acid positions, representing the TCR recognition site, were used in this thesis. The APLs with substitutions at position 26 (p.C26M, p.C26S, p.C26W) have immunomodulatory functions as partial agonists for allergen-specific T cells decreasing the production of IL-4, IL-5 and IL-13, while the APL generated by modification at position 28 (p.E28R) acts as an antagonistic control (Candia et al., unpublished work).

1.6 Virus-like nanoparticles

Virus-like nanoparticles (VNPs) are enveloped virus-like particles secreted by mammalian cells through the expression of viral structural proteins (Gag). Due to their lack of nucleic acid and envelope proteins, they are non-infectious and suitable allergen carriers for preventive vaccinations against different allergies or for therapeutic use in AIT. Kratzer et al. proved the

hypoallergenicity of the VNP itself whilst encasing Art v 1 allergen (Kratzer et al. 2019). Even though the allergenicity is reduced with surface-expressed allergen on VNP, they can still be allergenic and cause an anaphylactic reaction when given to sensitized patients. Whereas allergens linked to the Moloney Murine Leukemia Virus (MoMLV) MA p15 protein targeting the inner leaflet of the lipid bilayer envelope of the VNP, can bypass IgE cross-linking on sensitized mast cells, whilst maintaining or even enhancing the immunogenicity for presentation by APC to activate allergen-specific $CD4^+$ T cells, stimulate differentiation and secrete different cytokine profile. In addition, prophylactic administration of allergen shielding VNPs was able to increase the amount of Treg cells in allergen-specific mice and therefore suppress Th2 cell cytokine production when further challenged with the allergen. It has also been confirmed that the allergen in the VNP is crucial for achieving the effects of decreasing IL-2, IL-4, IL-5 and IL-13 levels and increasing Treg cells upon rechallenge in comparison to empty VNP (Schmitz et al. 2009, Kueng et al. 2010, Campana et al. 2011, Engeroff et al. 2018, Kratzer et al. 2019).

1.7 Interleukin-15

Cytokines have been known for a long time for having an important impact on the determination of different immune responses through activation and stimulation of differentiation of T-cell populations, depending on the type and concentration of these molecules in the microenvironment. Cytokines can be administrated as soluble molecules or as antibody-cytokine fusion proteins, so-called immunocytokines. Another option is anchoring the cytokines to other particles, like VNP. In my thesis, I have used, in particular, murine IL-15 (mIL-15) decorating the surface of VNPs. In general, functions of IL-15 include the activation of natural killer cells (NK cells), $CD8^+$ effector memory and central memory T cells. In the context of allergy, it has been reported that lack of IL-15 promotes basal airway resistance in mice while the delivery of soluble IL-15 promotes airway hyperresponsiveness. However, another study found that IL-15 deficient mice are more susceptible to Th2-mediated allergic airway diseases which also leads to deficiency of NK cells, natural killer T cells and their subsets. Therefore, it could be a superior adjuvant for vaccine strategies against infectious diseases for improving their effectiveness or used in allergen-specific immunotherapy to enhance/modify the activities of effector cell populations (Perera et al. 2012, Mathias et al. 2017).

In our lab, Sehgal et al. (unpublished work) recently demonstrated that MA::Art v 1 containing VNPs with surface-expressed murine IL-15 (mIL-15) significantly stimulated splenocytes from Art v 1-specific TCR/DR1 double transgenic humanized allergy mice compared to the unchaperoned MA::Art v 1 VNP. In addition, mIL-15 decorated VNP encasing Art v 1 also

showed a five-fold increase in the induced IFN-γ/IL-4-ratio together with a notable suppression of IL-5 and IL-13 cytokines in comparison to the non-decorated MA::Art v 1 VNP, implying a favourable Th1 shift.

1.8 Aim of the thesis

This thesis aims to produce and characterize mutated versions, so-called APL of full-length Art v 1 safely encased in VNP with surface-expressed mIL-15 (**Figure 1**) to evaluate their combined immunomodulatory effect in shifting the pathognomonic Th2 cytokine profile during allergic reactions to a potentially protective Th1 and/or T reg response.

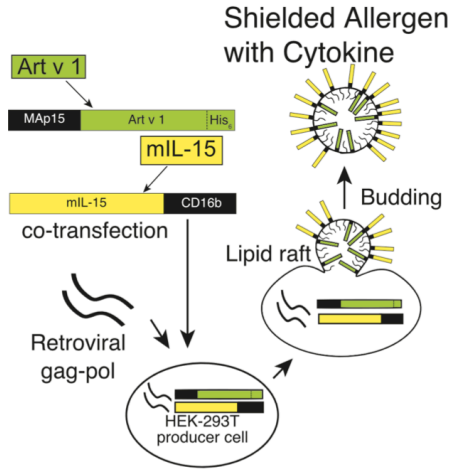


Figure 1: For the production of shielded Art v 1 inside VNPs, an N-terminal fusion of the Art v 1 to MAp15 of MoMLV and C-terminal his tag was designed. In addition, C-terminal fusion of the minimal CD16b GPI anchor acceptor sequence to the mIL-15 cytokine leads to the surface expression on HEK 293T producer cells and subsequently on emerging VNPs.

2. Material and Methods

2.1 Transformation of Escherichia coli with the heat shock method and preparation of miniprep-cultures

For the transformation of competent *Escherichia coli (E. coli*) MC1061, 5 µl of ligation mixture (provided by Al Nasar Ahmed Sehgal, M.Sc.) was added to 100 µl of freshly thawed *E. coli* cells and incubated on ice for 30 min. After incubation, the heat shock was performed at 42 °C in a heating block for 45 s. (Eppendorf Thermomixer C, Eppendorf, Germany). Subsequently, the mixture was transferred onto prewarmed agar dishes (32 g LB Agar Lennox, 5 g NaCl, 3 g Bacto-Agar, 5 mg/L Ampicillin) and plated with clean and autoclaved glass beads (Carl Roth GmbH + Co. KG, Germany) and careful shaking until evenly spread and dry. The plates were then incubated at 37 °C overnight (CO_2-AUTO-ZERO, Heraeus, Germany).

2.2 Preparation of miniprep-cultures of transformed Escherichia coli

Miniprep cultures of transformed *E. coli* colonies were inoculated in 5 ml LB medium with Ampicillin (5 mg/L) into autoclaved glass eprouvettes. Distinct and well-isolated colonies were then picked with a sterile pipette tip and transferred into the glass eprouvettes with one colony per eprouvette and incubated whilst shaking at 200 revolutions per minute (rpm) at 37 °C overnight (New Brunswick™ Innova40, Eppendorf, Germany). The isolation of the plasmid DNA was performed with the NucleoSpin Plasmid Kit (Macherey-Nagel, Germany) as per the manufacturer's protocol. The extraction included cell lysis, clarification, binding of the plasmid DNA to the membrane, washing steps and elution. Afterwards, it was verified with restriction digestion and DNA sequencing as described in section 2.5.

2.3 DNA-extraction with caesium chloride density-gradient method

For the extraction of bulk plasmid DNA from the *E. coli* cultures, the caesium chloride density-gradient method was employed. At first, 1 ml from the miniprep *E. coli* cultures was inoculated into 1 L LB medium (20 g Luria Broth (Invitrogen, Carlsbad, California), 5 g sodium chloride (Carl Roth GmbH + Co. KG, Germany)) and 500 µl Ampicillin (5 mg/L) in 2 L Erlenmeyer flasks (Schott, Germany) and incubated whilst constant agitation at 37 °C overnight (Multitron Shaker, Infors HT, Switzerland). The next day, the *E. coli* suspension was transferred into 1 L J6 bottles and centrifuged at 4.200 rpm at 4 °C for 20 min (Sorvall RC 3PLUS, Thermo Fisher Scientific Inc., Waltham, Massachusetts). After discarding the supernatant, the bacterial

cultures were resuspended in 40 ml Solution I (10 mmol/l EDTA, pH 8) and lysed by adding 80 ml of prewarmed, at 37°C, Solution II (0.1 M sodium hydroxide, 1 % sodium dodecyl sulfate (SDS)). The reaction was then neutralized by adding 40 ml of Solution III (5 M KOAc, pH = 4.7) (2.5 M KOAc, 2.5 M HOAc) and the cellular remnants of the lysed bacteria were spun down at 4200 rpm at 4 °C for 5 min (Sorvall RC 3PLUS, Thermo Fisher Scientific Inc., Waltham, Massachusetts). Next, the DNA-containing supernatants were filtered through a gauze compress (Lohmann-Rauscher Gazin, Austria) into 250 ml bottles. Afterwards, the bottles were filled up with Isopropanol alcohol (2-propanol, Carl Roth GmbH + Co. KG, Germany) and centrifuged at 4.200 rpm at 4 °C for 10 min (Sorvall RC 3PLUS, Thermo Fisher Scientific Inc., Waltham, Massachusetts). After discarding the supernatant, the DNA pellets were carefully rinsed with 5 ml of 70 % ethanol, air-dried for at least one hour, until the pellets turned transparent, and then resuspended in 4.2 ml of 10 mM ethylenediaminetetraacetic acid (EDTA). In the next step, 4.4 ml of each resuspended pellet, 5.5 g of powdered caesium chloride, 0.1 ml of 1 % Triton X-100 and 0.5 ml ethidium bromide (10 mg/ml) were added to 14 ml Sarstedt-tubes with caps (Sarstedt, Germany) and agitated at 180 rpm at 37 °C for at least 30 min (Multitron Shaker, Infors HT, Swiss). For removing the insoluble precipitates, the tubes were centrifuged at 10.000 rpm at 25 °C for 10 min (Sorvall Lynx 4000 Centrifuge, Thermo Fisher Scientific Inc., Waltham, Massachusetts). Afterwards, polyallomer quick-seal tubes (Beckman Coulter Inc., Brea, California) were filled with the plasmid-containing clear solution and sealed with a tube sealer (Beckman Coulter Inc., Brea, California). The next step was ultracentrifugation at 68000 rpm at 25 °C for 16 hours (acceleration = 1, deceleration = no brake, rotor: NVT100, centrifuge: Beckman Coulter Optima L100XP Ultracentrifuge, Beckman Coulter Inc., Brea, California). After the ultracentrifugation, there were two red bands visible in the tubes, the upper ones corresponded to the genomic DNA and/or small amounts of relaxed plasmid DNA, and the one below contained super-coiled plasmid DNA. The super-coiled plasmid DNA was taken out and transferred into a new 14 ml Sarstedt tube. To get rid of the DNA-bound ethidium bromide (EtBr), the tubes were filled up with n-butanol saturated with 1 M NaCl, mixed, and after the separation of the two phases, the supernatant was aspirated with a Pasteur glass pipette using a suction pump. These steps were repeated until the DNA-containing lower phase was transparent and free of ethidium bromide. Under sterile conditions, an equal volume of 1 M ammonium acetate as obtained DNA (1–1,5 ml) and 96 % ethanol two times as much the volume was added to the samples. After centrifugation at 10000 rpm at 4 °C for 10 min (Sorvall Lynx 4000 Centrifuge, Thermo Fisher Scientific Inc., Waltham, Massachusetts), the supernatant was removed and air dried in a sterile environment until the

DNA pellet turned transparent. In the end, the pellets were resuspended in 500 µl sterile 1X Tris-EDTA-buffer (1X TE-buffer) (pH = 8.0), left at 4 °C overnight, transferred into a sterile 1.5 ml Eppendorf tube (Eppendorf, Germany) and stored at 4 °C.

2.4 Measurement of DNA concentration with Nanodrop

The concentration of the extracted super-coiled DNA plasmid was determined with the Nanodrop 2000 Spectrophotometer (Thermo Scientific Fisher Inc., Waltham, Massachusetts) by measuring the absorbance at 260 nm. Five µl of the DNA solution was diluted 1:10 in 1X TE buffer and measured after compensating against the blank (1X TE buffer).

2.5 Verification of isolated plasmid DNA with restriction enzyme digestion

For the diagnostic restriction digestion, the obtained plasmid DNA samples were digested with XhoI (New England BioLabs GmbH, Ipswich, Massachusettes) and NotI-HF (New England BioLabs GmbH, Ipswich, Massachusettes) restriction enzymes in the compatible CutSmart buffer (New England BioLabs GmbH, Ipswich, Massachusettes). The digestion reaction was prepared with the recipe as shown in Fehler! Verweisquelle konnte nicht gefunden werden. and incubated at 37 °C for at least 2 hours and at most overnight.

For screening the digested DNA bands, 1 % agarose (Biozym LE agarose, LOT: 0000648796) gel was prepared in 1X Tris-acetate-EDTA (TAE)-buffer by heating in the microwave with periodic agitation for 3–4 minutes. Afterwards, 4 µl of 10 mg/ml EtBr per 100 ml of gel was added (final concentration of EtBr used = 0.4 µg/ml), and the solution was transferred into the casting tray for solidifying. Depending on the number of samples, the small cast with the small gel chamber (Mini-Sub Cell-GT, Bio-Rad, Hercules, California) was used for eight or fewer samples, and a big cast with the big gel chamber (Wide Mini-Sub Cell-GT) for up to 20 samples. After solidifying, the casting tray containing the gel was put into an electrophoresis chamber and filled with 1X TAE buffer. In the next step, 1X loading dye (Gel Loading Dye, Purple (6X), New England Bio Labs Inc.) was added to the samples. Following, the complete sample volume and 12 µl DNA marker mix (pBR322 DNA-MspI Digest, size range: 117 bp–8,454 bp and λ DNA-BstEII Digest, size range: 9 bp–622 bp) were loaded into the wells of the gel respectively. The electrophoresis was then performed at 90 V with a continuous current for 90 min for the small gel and at 95 V with a continuous current for 95 min for the big gel (PS300-B, Hoefer Inc., Holliston, Massachusetts). After the run, the gel was visualized in the gel document imaging system (Quantum, Vilber) at 365 nm ultraviolet (UV) light and the size of the digested fragments was determined by comparing their position to the ladder bands.

Table 1: Recipe for control restriction digestion for one reaction, 30 µl

Recipe for a single 30 µl control restriction digestion reaction		**Company**
1 µg	plasmid DNA	
3 µl	CutSmart buffer	New England Biolabs, GmbH
0.5 µl	XhoI	New England Biolabs, GmbH
0.5 µl	NotI-HF	New England Biolabs, GmbH
Fill up to 30 µl	UltraPure™ DNase/RNase-free water	Invitrogen, Carlsbad, California

2.6 DNA sequencing analysis of isolated plasmid DNA

The next step in the verification of the plasmid DNA was Sanger sequencing which was performed by Eurofins Genomics. The sample for sequencing was prepared as follows and sent to the company; 1000 ng DNA, DNase/RNase-free water up to 15 µl, and 2 µl of vector-specific pk12-F forward primer (5' CATTCTCAAGCCTCAGACAGTGG 3', Sigma-Aldrich Chemie GmbH).

The nucleotide sequence from the sequence trace file (.scf) was aligned with the known plasmid sequence of his-tagged MA::Art v 1 using the Clustal Omega Multiple Sequence Alignment tool (Madeira et al. 2022). In the comparison, the nucleotide codons for the 26th or 28th amino acid position and the fusion of his_6-tag at the C-terminus were precisely checked.

2.7 Cell culture

In this project, lab-isolates of human embryonic kidney 293T (H293T) cells were cultivated in adherent T75 flasks (Cellstar Cell Culture Flasks, Greiner Bio-One GmbH, Germany) in an IMDM-modified medium (Cytiva, Germany) with already added 4 mM L-Glutamine and HEPES. In addition, 10 % fetal bovine serum (FBS) (Gibco, Thermo Scientific Fisher Inc., Waltham, Massachusetts) and 150 µl gentamicin (50 mg/ml, Gibco) were added to the medium.

For cell passaging, the old medium in the flask was replaced with a 4 ml 0.05 % Trypsin-EDTA solution (Gibco, Fisher Scientific) for detachment of the cells from the flask. After incubating the flask at 37 °C for 5 min (Forma Steri-Cult CO_2-Incubator, Thermo Scientific Fisher Inc., Waltham, Massachusetts), the cell suspension was spilt against the wall of the flask with a 5 ml pipette to dislodge any leftover cells and then was transferred into a 50 ml Falcon tube

(Sarstedt, Germany) together with 20 ml medium to inhibit further trypsin digestion. Next, a centrifugation step at 1542 rpm at 20 °C for 5 min (Sigma 4-16KS, Sigma Laborzentrifugen GmbH, Germany) was performed, taking off the supernatant and efficiently resuspending the cell pellet in a fresh 5 ml medium.

For counting cells, 20 µl of the cell suspension was added to a 10 ml Coulter-Isoton II isotonic solution in a Coulter counter beaker (Beckman Coulter Inc., Brea, California), and cell count was determined using the Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter Inc., Brea, California) with the profile E set at the particle size between 8 µm and 22 µm.

The cell line was maintained by seeding at a density of 1 million cells in a 29 ml medium in T75 flasks for 3 days, or 0.5 million cells for 4 days of propagation, and incubated at 37 °C in a humidified atmosphere (95 % humidity) and 5 % carbon dioxide (CO_2).

2.8 Transient transfection of H293T cells

For the transfection of H293T cells with the required plasmid, the day before the transfection, $1x10^6$ cells were seeded in a 10 ml IMDM medium in a culture dish for adherent cells (TC Dish 100, Standard, Sarstedt, Germany) and incubated at 37 °C (Forma Steri-Cult CO_2-Incubator, Thermo Scientific Fisher Inc., Waltham, Massachusetts). On the day of the transfection, 2–3 hours before, the medium was replaced with 8 ml of fresh medium. 30 µg of plasmid DNA, DNase/RNase-free water up to 900 µl, and 100 µl 2.5 M $CaCl_2$ were added in a 50 ml Falcon tube (Sarstedt, Germany). While vortexing, 1 ml 2X HBS buffer was slowly added into the tube and incubated at room temperature for 1 min. The solution was then added to the cells in the dish drop-wise and the dish was slightly agitated at the end. Afterwards, the plate was incubated at 37 °C overnight. The next day, the medium was again exchanged. Forty-eight hours after the transfection, the medium was removed and cells were harvested after detaching with 5 ml 1X Phosphate-buffered saline (PBS) containing 2 mM EDTA. After an incubation at room temperature for 1 min, the cell suspension was transferred from the dish into a 15 ml Falcon tube (Sarstedt, Germany). The cell pellet was washed with 1X PBS after centrifugation at 500 g at 20 °C for 5 min (Heraeus Multifuge X3R, Thermo Fisher Scientific Inc., Waltham, Massachusetts) and the process was repeated three times to remove any traces of medium. After washing, the cell pellet was resuspended in 3–5 ml 1X PBS, depending on the pellet size, and the cell suspension was counted as described above.

2.9 Intracellular and extracellular flow cytometry staining

To the PBS-washed cell suspension of transfected cells, 0.1 µl Zombie Aqua™ Dye in DMSO (Zombie Aqua™ Fixable Viability Kit, BioLegend, San Diego, California) was added per 0.5 x 10^6 cells and incubated at room temperature in the dark for 10 min. To stop the reaction, the cells were washed with Fluorescence Activated Cell Sorting buffer (FACS buffer) (1X PBS, 0.5 % bovine serum albumin (BSA), 0.05 % sodium azide and 2 mM EDTA) by centrifugation at 500 g at 20 °C for 5 min (Heraeus Multifuge X3R, Thermo Fisher Scientific Inc., Waltham, Massachusetts). Afterwards, the cells were resuspended in 50 µl FACS buffer per 0.5 x 10^6 cells. Fifty µl (0.5 x 10^6) of cells were transferred into each FACS tube (5 ml Polystyrene Round-Bottom Tube, Corning, Corning, New York) for each staining. For intracellular staining, 500 µl of fixation buffer (BioLegend, San Diego, California) was added to each tube and then incubated at room temperature in the dark for 20 min. After incubation, the tubes were centrifuged at 1542 rpm at 4 °C for 5 min (Heraeus Multifuge X3R, Thermo Fisher Scientific Inc., Waltham, Massachusetts). The supernatant was discarded, and the cells were washed twice with 1X Intracellular Staining Perm Wash Buffer, (10x Intracellular Staining Perm Wash Buffer, BioLegend, San Diego, California). The cells were probed with 20 µl of antibodies (below) diluted in 1X Intracellular Staining Perm Wash Buffer and incubated in the dark at 4 °C for 30 min. For the detection of the his-tag, a phycoerythrin (PE)-conjugated mouse anti-his tag IgG2 (clone: J095G46, BioLegend, San Diego, California) diluted 1:50 was used and for the indirect staining of Art v 1, a rabbit anti-Art v 1-P4 anti-serum followed by PE-conjugated affinipure F(ab')2 fragment goat anti-Rabbit IgG (H+L) (Jackson ImmuneResearch Laboratories Inc., United Kingdom) as a secondary antibody, both diluted 1:100 for detection of Art v 1 was used. After each staining, the cells were washed twice with 1X Intracellular Staining Perm Wash Buffer and in the end once with FACS buffer with centrifugation settings at 1542 rpm at 20 °C for 5 min (Heraeus Multifuge X3R, Thermo Fisher Scientific Inc., Waltham, Massachusetts). The extracellular staining of the pk12-mIL-15-GPI-transfected cells was performed with a polyclonal goat anti-mouse IL-15 IgG antibody (R&D systems, Minneapolis, Minnesota), diluted 1:5 in FACS buffer and PE-conjugated affinipure F(ab')2 fragment donkey anti-goat IgG (H+L) (Jackson ImmuneResearch Laboratories Inc., United Kingdom) in a 1:50 dilution in FACS buffer with 30 minutes of incubation at 4 °C for each step and washing away the unbound antibody with FACS buffer at 1542 rpm at 20 °C for 5 min in the centrifuge (Heraeus Multifuge X3R, Thermo Fisher Scientific Inc., Waltham, Massachusetts). The applied antibodies are also listed in **Table 5** for a better overview.

The measurement of the stained H293T cells was performed with a Cytoflex (Beckman Coulter Inc., Brea, California) flow cytometer. The staining with Aqua Zombie was detected at 525 nm ± 40 nm (KO525-A channel) upon excitation with the 405 nm laser and the antibody staining was detected at 585 nm ± 42 nm (PE-A channel) upon excitation with the 488 nm laser. The data were analysed with FlowJo™ version 10.7.0 Software (Becton Dickinson, 2021) and for statistical analysis with a Kruskal-Wallis for non-parametric data using GraphPad Prism version 9.4.0 for Mac. (“GraphPad Software” 2022)

2.10 VNP-production and purification

On the first day, 3.5 million H293T cells were seeded in 15 cm culture dishes for adherent cell growth (TC Dish 100, Standard, Sarstedt, Germany) with the 25 ml IMDM Modified medium and incubated at 37 °C overnight (Forma Steri-Cult CO_2-Incubator, Thermo Scientific Fisher Inc., Waltham, Massachusetts). Five plates were seeded for each VNP set.

The following day, the cells were transfected with the three necessary plasmids for each VNP set, pMD-OGP (containing a *gag-pol* sequence of Moloney Murine Leukemia Virus (MoMLV), with the information for VNP budding), pk12-MA::Art v 1 (Art v 1 fused to the MoMLV matrix protein, MAp15, to express Art v 1 within the VNP or APL version of Art v 1) and pk12-mIL-15-GPI (mIL-15 with a CD16b GPI-anchor attachment sequence). The required volume of each plasmid DNA (15 µg each) and DNase/RNase-free water up to a total volume of 13.5 µl were added into a 50 ml Falcon tube and are listed in Table 3. Then, 1.5 ml 2.5 M $CaCl_2$ was added and 15 ml 2X HBS buffer was slowly added while vortexing. After incubation at room temperature for one minute, 6 ml of the solution was added dropwise onto each culture dish and incubated at 37 °C overnight. On the third day, the medium was exchanged with 25 ml fresh medium and incubated at 37 °C again.

Seventy-two hours after the transfection, the medium containing the produced VNPs was harvested, transferred into 50 ml Falcon tubes and centrifuged at 1542 rpm at 4 °C for 5 min (Sigma 4-16KS, Sigma Laborzentrifugen GmbH, Germany) to get rid of any unwanted cell remnants and subsequentially filtered through 0.45 µm filter (Millipore, Billerica, MA). The concentration of the VNPs was performed in Centricons (Centricon Plus – 70, Sigma Aldrich, St.Louis, Missouri). Therefore, the Centricons were filled each with 70 ml of the supernatant and centrifuged at 3000 rpm at 20 °C for 40 min (Sorvall RC 3PLUS, Thermo Fisher Scientific Inc., Waltham, Massachusetts). The concentrated VNPs were eluted into the concentrate collection cup by centrifuging at 1000 rpm for 5 min. These two steps were repeated with the remaining 55 ml supernatant as well. Afterwards, the concentrated supernatant was

transferred into an ultracentrifuge tube (Polypropylene Centrifuge Tubes, Beckman Coulter Inc., Brea, California) and filled with 1X PBS with added calcium and magnesium ions (1X PBS^{++}). The ultracentrifugation was performed at 28000 rpm at 4 °C for 1 hour (acceleration = max., deceleration = max.) (rotor: SW 41, centrifuge: Beckman Coulter Optima L100XP Ultracentrifuge, Beckman Coulter Inc., Brea, California). This washing step was repeated three times. Afterwards, the VNP-containing pellet was resuspended in 200–400 µl 1X PBS^{++}, covered with Parafilm (Sigma Aldrich, St.Louis, Missouri) and stored at 4 °C for solubilizing overnight. On the next day, the solubilized VNPs were transferred into sterile 1.5 ml Eppendorf tubes and stored at 4 °C until use.

In the subsequent methods and results, the following VNP preparations were characterised, analysed, and further referred to as listed in **Table 2**.

Table 2: List of the analyzed VNP preparations composed of the following plasmids and their further used abbreviations.

VNP preparations containing the following plasmids	**Further used Abbreviation**
pk12-poly	poly VNP
pk12-MA::Art v 1	WT VNP
pk12-MA::Art v 1 + pk12-mIL-15-GPI	WT + mIL-15 VNP
pk12-MA::Art v 1 (C26M)	C26M VNP
pk12-MA::Art v 1 (C26M) + pk12-mIL-15-GPI	C26M + mIL-15 VNP
pk12-MA::Art v 1 (C26S)	C26S VNP
pk12-MA::Art v 1 (C26S) + pk12-mIL-15-GPI	C26S + mIL-15 VNP
pk12-MA::Art v 1 (C26W)	C26W VNP
pk12-MA::Art v 1 (C26W) + pk12-mIL-15-GPI	C26W + mIL-15 VNP
pk12-MA::Art v 1 (E28R)	E28R VNP
pk12-MA::Art v 1 (E28R) + pk12-mIL-15-GPI	E28R + mIL-15 VNP
pk12-poly + pk12-mIL-15-GPI	poly + mIL-15 VNP

Table 3: Calculations of the required volume of the different plasmids for VNP production and the additional amount of DNase/RNase-free water for a total volume of 13.5 µl.

Nr.	VNP preparation	Plasmid	Amount [µg]	Concentration [µg/µl]	Volume used [µl]	Volume of DNase/RNase-free water [ml]
1	**poly VNP**	pk12-poly	300	3,56	84	13,348
		pMD-OGP	150	2,21	68	
2	**WT VNP**	pk12-poly	150	3,56	42	13,353
		pk12-MA::Art v 1-His	150	4,1	37	
		pMD-OGP	150	2,21	68	
3	**WT + mIL-15 VNP**	pk12 MA::Art v 1-His	150	4,1	37	13,320
		pk12-mIL15-GPI	150	1,99	75	
		pMD-OGP	150	2,21	68	
4	**C26M VNP**	pk12-poly	150	2,046	73	13,319
		pMD-OGP	150	1,976	76	
		pk12-MA::Art v 1-His (C26M)	150	4,749	32	
5	**C26M + mIL-15 VNP**	pk12-mIL-15-GPI	150	1,578	95	13,297
		pMD-OGP	150	1,976	76	
		pk12-MA::Art v 1-His (C26M)	150	4,749	32	
6	**C26S VNP**	pk12-poly	150	2,046	73	13,289
		pMD-OGP	150	1,976	76	
		pk12-MA::Art v 1-His (C26S)	150	2,419	62	

7	**C26S + mIL-15 VNP**	pk12-mIL-15-GPI	150	1,578	95	13,267
		pMD-OGP	150	1,976	76	
		pk12-MA::Art v 1-His (C26S)	150	2,419	62	
8	**C26W VNP**	pk12-poly	150	2,046	73	13,307
		pMD-OGP	150	1,976	76	
		pk12-MA::Art v 1-His (C26W)	150	3,447	44	
9	**C26W + mIL-15 VNP**	pk12-mIL-15-GPI	150	1,578	95	13,285
		pMD-OGP	150	1,976	76	
		pk12-MA::Art v 1-His (C26W)	150	3,447	44	
10	**E28R VNP**	pk12-poly	150	2,726	55	13,289
		pMD-OGP	150	2,077	72	
		pk12-MA::Art v 1-His (E28R)	150	1,777	84	
11	**E28R + mIL-15 VNP**	pk12-mIL-15-GPI	150	1,985	76	13,268
		pMD-OGP	150	2,077	72	
		pk12-MA::Art v 1-His (E28R)	150	1,777	84	
12	**poly + mIL-15 VNP**	pk12-poly	150	3,56	42	13,315
		pk12-mIL15-GPI	150	1,99	75	
		pMD-OGP	150	2,21	68	

2.11 Micro BCA Protein Assay

The total protein quantification was performed with Micro BCA™ Protein Assay Kit (Thermo Scientific Fisher Inc., Waltham, Massachusetts), with the microplate procedure for a linear working range of 2–40 µg/ml and carried out in a flat 96-well plate (BRANDPLATES®, pureGRADE). At first, the bovine serum albumin (BSA) standard dilutions (200, 40, 20. 10, 5, 2.5, 1, 0.5 µg/ml) were prepared from BSA standard ampules with 2 mg/ml in the kit diluted in 1X PBS^{++}. The samples were diluted at 1:50, 1:100, and 1:200 also in 1X PBS^{++}. Then, 150 µl of each standard and sample was added to the plate, and for the blank 150 µl of 1X PBS++ was added like in the layout displayed in **Table 4**. Subsequently, for the working reagent 25 parts of Micro BCA Reagent MA, 24 parts of Micro BCA Reagent MB, and 1 part of Micro BCA Reagent MC were mixed and 150 µl of which was added to each well. The plate was placed on a plate shaker for 30 sec and then incubated at 37 °C for 2 hours (CO_2-Auto-Zero, Heraeus, Germany). After cooling down to room temperature the absorbance was measured at 562 nm with the Multiscan Go (Thermo Scientific Fisher Inc., Waltham, Massachusetts) and the SkanIt Plate Reader Software (SkanIt RE, Thermo Scientific Fisher Inc., Waltham, Massachusetts).

Table 4: Layout of the 96-well plate for the Micro Protein BCA assay for determination of the total protein amount with four VNP preparations per plate.
The applied BSA standards in concentrations of 200, 40, 20, 10, 5, 2.5, 1, and 0.5 µg/ml are shown in yellow, the wells with the blanks in green and the VNP preparations in dilutions of 1:50, 1:100 and 1:200 in blue. The standards and the VNP preparations were diluted in 1X PBS++, which was also used for the blank.

	1	2	3	4	5	6	7	8	9	10	11	12
A	200	40	20	10	5	2.5	1.00	0.50	blank			
B	200	40	20	10	5	2.5	1.00	0.50	blank			
C	200	40	20	10	5	2.5	1.00	0.50	blank			
D												
E	1:50	1:100	1:200	1:50	1:100	1:200	1:50	1:100	1:200	1:50	1:100	1:200
F	1:50	1:100	1:200	1:50	1:100	1:200	1:50	1:100	1:200	1:50	1:100	1:200
G	1:50	1:100	1:200	1:50	1:100	1:200	1:50	1:100	1:200	1:50	1:100	1:200
H												

2.12 Double-sandwich ELISA

At first, a half-area high-binding 96-well plate (Microlon, Greiner bio-one, Austria) was coated with 40 µl per well with an unconjugated mouse anti-his IgG1 antibody (clone 13/45/31,

1 mg/ml, Dianova, Germany) in a concentration of 1 µg/ml diluted in carbonate buffer (1.59 g/l Na_2CO_3, 2.93 g/l $NaHCO_3$, pH = 9.6, adjusted with 1 M HCl) at 4 °C overnight. On the next day, the plate was washed six times with 150 µl of 0.5 % BSA in 1X phosphate buffered saline with 0.1 % Tween20 (1X PBST). In the next step, the plate was blocked with 100 µl of 3 % BSA in 1X PBST at room temperature whilst gently shaking for 2 hours. After flicking the plate, 40 µl of the samples, standard dilutions and the negative control were added to respective wells. The samples were diluted in 0.5 % BSA in 1X PBST in concentrations of 3, 0.1 and 0.03 µg/ml. Afterwards, the plate was washed again as described above and the samples were detected with 40 µl of rabbit-anti-Art v 1-P4 anti-serum diluted 1:10000 in 0.5 % BSA in 1X PBST overnight and 40 µl of the detection antibody, horseradish peroxidase (HRP)-conjugated affinipure goat anti-rabbit IgG (H+L) (Jackson Immuno Research), in a 1:100000 dilution in 0.5 % BSA in 1X PBST incubated for 2 hours with a washing step in between and afterwards. All of the applied antibodies are also listed in **Table 5** for a better overview. In the last step, 40 µl tetramethylbenzidine (TMB) (3,3',5,5'-tetramethylbenzidine, Sigma Aldrich, St.Louis, Missouri) was added per well and incubated at room temperature until the least sample concentration showed blue color. The reaction was stopped by adding 30 µl 2 M HCl and instantly measured at 450 nm with the Multiscan Go (Thermo Scientific Fisher Inc., Waltham, Massachusetts) and the SkanIt Plate Reader Software (SkanIt RE, Thermo Scientific Fisher Inc., Waltham, Massachusetts). The measurements were analyzed with GraphPad Prism version 9.4.0 for Mac. ("GraphPad Software" 2022)

2.13 Western Blot

The SDS PAGE for the western blot was performed with a 4–18 % gradient polyacrylamide gel. For the 18 % resolving gel, 3.08 ml acrylamide (30 % Acrylamide Gel 30, Carl Roth GmbH + Co. KG, Germany), 1.875 ml of 1.5 M Tris buffer (pH = 8.8) with 0.1 % SDS, 50 µl ammonium persulfate (APS) and 4 µl N,N,N',N'-Tetramethylethylendiamin (TEMED) (Carl Roth GmbH + Co. KG, Germany) were mixed for a total of 5 ml. For the 4 % resolving gel, 0.67 ml acrylamide (30 % Acrylamide Gel 30, Carl Roth GmbH + Co. KG, Germany), 1.875 ml of 1.5 M Tris buffer (pH = 8.8) with 0.1 % SDS, 2.425 ml distilled vented water, 50 µl APS and 4 µl TEMED was mixed together. The separating gels were filled into the cast and left for solidifying. To reduce bubbles and flatten out the surface, 1 ml Isopropanol was added on top. Afterwards, the stacking gel was prepared as the 4 % parting gel above, but with 1.875 ml of 0.5 M Tris buffer (pH = 6.8) and added to the cast together with a 15-well comb. Subsequently to the polymerization of the stacking gel, the cast and the electrophoresis chamber (PerfectBlue™

Twin M, VWR, USA) were filled up with 1X Tris/Glycine Buffer (catalogue no. 1610734, Bio-Rad Laboratories). Each VNP sample was standardized for a final amount of 15 µg with 1X PBS^{++} up to 21 µl and 7 µl 1X reducing loading dye (stock concentration = 4X reducing loading dye, lab-made) per well. Following, the samples were heated at 95 °C for 5 min and 15 µl each was added into the wells. The ladder (PageRuler Plus Prestained Protein Ladder 10 to 250 kDa, Thermo Scientific Fisher Inc., Waltham, Massachusetts) was added with 10 µl per well and the gel was run at 100 V with a continuous current in the beginning and turned up 20 V every 30 min. The electrophoresis was stopped once the samples had nearly migrated through the gel.

For the electrophoretic transfer of the proteins onto the polyvinylidene difluoride (PVDF) membrane, six filters (3 mm chromatography paper, Whatman, Great Britain) and one PVDF membrane (Immun-Blot® PVDF Membranes for Protein Blotting, Bio-Rad, Hercules, California) were cut in the size of the separating gel. Before plotting, the filters were soaked in 1X Western Blot buffer (3.02 g Tris, 14.4 g glycine up to 1 l) with 20 % ethanol for 5 min and the membrane was charged in absolute ethanol (VWR International LTD, Great Britain) for 1 min. In the next step, all the components were put together as shown in **Figure 2** and resolved proteins were blotted onto the PVDF membrane performed with 1.5 mA/cm^2 for 60 min.

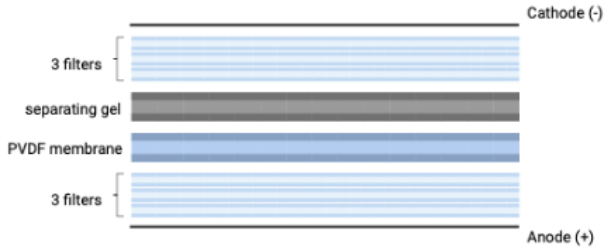


Figure 2: Composition of components for electrophoretic transfer from the separating gel onto the PVDF membrane

The immunostaining was executed with mouse anti-his IgG1 antibody (1:1000) (clone 13/45/31, 1 mg/ml, Dianova, Germany) followed by ECL HRP-linked sheep anti-mouse whole antibody IgG (1:10000) (NA931V, Cytiva, Germany) for the detection of the VNPs encasing his-tagged APLs. Afterwards, the membrane was stained with anti-p30gag rat sera (1:10) followed by ECL HRP-linked goat anti-rat whole antibody IgG (1:5000) (NA935V, Cytiva, Germany) for detection of the p30 gag protein incorporated into the VNPs and with poly ("empty") VNP constructs serving as positive controls. All antibodies were diluted in 0.5 % BSA in 1X Tris-buffered saline with 0.1 % Tween20 (TBST) and are also listed in **Table 5** for a better

overview. At first, the membranes were blocked with 3 % BSA in 1X TBST for 2–3 h. Followed by incubation with the primary antibody overnight, washing three times with 1X TBST for 5 min and incubation of the secondary antibody for 1–2 h. Subsequently, the membrane was washed three times again and the chemiluminescence of the reaction between the detection antibodies and the substrate (Clarity^{TM} Western ECL Substrate, Bio-Rad, Hercules, California) was measured with automatic exposure time with Fuji (Luminescent Image Analyzer, LAS-4000, Japan). Pictures were also taken at the half and twice of the automatic exposure time, and even longer, until the signal was oversaturated. After the measurement of the anti-his staining, the membrane was stripped three times with stripping buffer for 10 minutes, to get rid of all the bound secondary antibodies on the membranes. Next, the membrane was again blocked, incubated with primary and secondary antibodies for the detection of p30gag, washed and measured again.

2.14 Rat basophil leukemia cell-based immunological assay

The hypoallergenicity of the VNP preparations was tested on rat basophil leukemia cells (RBL cells). A β-hexosaminidase release test was performed with different controls for the determination of the percentage of β-hexosaminidase released. The cells were incubated with a 1 % final concentration of Triton X-100 for inducing 100 % release, 10 µg/ml anti-mouse IgE mAb (clone: R35-72, BD-Pharmingen) as positive control and recombinant Art v 1 (rArt v 1) in concentrations of 0.01, 0.1, 1 and 10 µg/ml. RBL cells were seeded with 6x 10^5 cells/well in 100 µl RPMI-1640 medium + 2.05 mM L-Glutamine (Cytiva, Germany) (with additionally added 10 % FBS (Gibco, Thermo Scientific Fisher Inc., Waltham, Massachusetts), 150 µl gentamycin and 1X Pen/Strep/Amp solution and incubated at 37 °C with 7 % CO_2 overnight. In the next step, 3 µl of Art v 1-specific IgE-containing mouse serum was added to each well to sensitize the RBL cells and incubated under the same conditions for 2 hours. Afterwards, the cells were washed twice with Tyrode/BSA buffer (9.5 g Tyrode salts (Gibco), 2.38 g HEPES, 1 g NaHCO3, adjusted to pH 7.2 with 5 M NaOH and 0.1 % BSA was added right before use) before adding 100 µl of antigen solution (standards, VNP preparations, anti-IgE) diluted in Tyrode/BSA buffer. The VNP preparations were added in final concentrations of 0.1, 1, 10 and 100 µg/ml of the total protein amount. After another hour of incubation at room temperature, the Triton X-100 was added and resuspended with the cells. Finally, assay solution (5 ml citrate buffer (0.1 M citric acid solution with pH 4.5) and 80 µl 4-MUG (10 mM stock solution in DMSO with 3.79 mg/ml, stored at -80 °C) was added 1:1 to the stimulated cells. After another hour of

incubation at room temperature, the reaction was stopped by adding glycine buffer 1:1 and measured with excitation at 355 nm and emission at 465 nm and a manual gain at 60.

Table 5: Overview of the antibodies used in the different experiments with their clone names, isotypes, names of the respective company distributors, used dilutions and methods.

Nr.	Name	Clone	Isotype	Company	Dilution	Purpose
1	PE-conjugated mouse anti-his tag IgG2a	J095G46	IgG2a	BioLegend, San Diego, California	1:50	Intracellular flow cytometry staining
2	Rabbit anti-Art v 1 P-4 anti-serum	-	-	Zabel et al. 2022	1:100	Intracellular flow cytometry staining
					1:1000	ELISA
3	PE-conjugated affinipure F(ab‘)2 fragment goat anti-rabbit IgG (H+L)	-	IgG	Jackson ImmuneResearch Laboratories Inc., United Kingdom	1:100	Intracellular flow cytometry staining
4	Polyclonal goat anti-mouse IL-15 IgG	-	IgG	R&D systems, Minneapolis, Minnesota	1:5	extracellular flow cytometry staining
5	PE-conjugated affinipure F(ab’)2 fragment donkey anti-goat IgG (H+L)	-	IgG	Jackson ImmuneResearch Laboratories Inc., United Kingdom	1:50	extracellular flow cytometry staining

6	Unconjugated mouse anti-his IgG1	13/45/31	IgG	Dianova, Germany	1:1000	ELISA
7	HRP-conjugated affinipure goat anti-rabbit IgG (H+L)	-	IgG	Jackson ImmuneResearch Laboratories Inc., United Kingdom	1:10000	ELISA
8	ECL HRP-conjugated sheep anti-mouse IgG	NA931V	IgG	Cytiva, Germany	1:10000	Western Blot
9	Rat anti-p30gag sera	-	-	Provided by R. Weiss, Salzburg, AUT	1:10	Western Blot
10	ECL HRP-conjugated goat anti-rat IgG	NA935V	IgG	Cytiva, Germany	1:5000	Western Blot
11	Unconjugated anti-mouse IgE mAb	R35-72	IgE	BD-Pharmingen	1:50	RBL cell-based immunological assay

3. Results

3.1 Concentration of extracted plasmid DNA

In a first step, the plasmid DNAs required for generating the VNPs used in this study had to be prepared. The APL versions of the different Art v 1 expression constructs were designed as His-tagged proteins. The results of the 1:10 diluted nucleic acid concentration measurement and the total amounts in 500 µl of obtained plasmid DNAs in 1X TE-buffer from the DNA extraction exerpiments with the caesium chloride density-gradient method are listed in **Table 6** in ng/µl. The obtained plasmid amounts ranged from 0.249 mg to 2.375 mg with an SD of 375.5 µg. The 260/280 and 260/230 ratios determine the purity of the measured nucleic acid, due to maximal absorbance of DNA at 260 nm and proteins at 280 nm and various organic contaminants at 230 nm. The 260/280 ratio should give an output of around 1.8 for pure DNA and the 260/230 ratio between 2.0–2.2 for DNA. (Assessment of Nucleic Acid Purity 2011) The results of the 260/280 ratio are ranging from 1.9 to 2.04. With the 260/230 ratio, the values are between 2.24–2.41.

Table 6: Measurement of nucleic concentrations in ng/µl with the Nanodrop 2000 Spectrophotometer of the plasmid DNA samples extracted with the caesium chloride density-gradient method.
The obtained plasmids are pk12-poly, pMD-OGP, pk12-MA::Art v 1-His & APLs (C26M, C26S, C26W, E28R) and the cytokine pk12-mIL-15-GPI. The total amounts of obtained plasmid DNA in 500 µl 1X TE-buffer are displayed and the absorbances measured at 230 nm, 260 nm and 280 nm wavelengths were used to calculate "260/280" and "260/230" ratios inorder to evaluate the purity of the nucleic acid.

Plasmid DNA	Nucleic acid conc. [ng/µl] (diluted 1:10)	Total amount extracted [mg]	260/280 ratio	260/230 ratio
pk12-poly 1	204.6	1.023	1.93	2.31
pk12-poly 2	49.8	0.249	1.92	2.40
pk12-poly 3	272.6	1.363	1.98	2.28
pk12-poly 4	356.2	1.781	1.93	2.27
pk12-poly 5	107.4	0.537	1.92	2.41
pMD-OGP 1	221.3	1.107	1.94	2.34
pMD-OGP 2	197.6	0.988	1.95	2.34
pMD-OGP 3	207.7	1.039	1.94	2.37

pk12-MA::Art v 1-His (C26M) 1	474.9	2.375	1.93	2.24
pk12-MA::Art v 1-His (C26M) 2	294.9	1.475	1.93	2.31
pk12-MA::Art v 1-His (C26S) 1	241.9	1.210	2.04	2.28
pk12-MA::Art v 1-His (C26S) 2	308.0	1.540	1.92	2.28
pk12-MA::Art v 1-His (C26W) 1	344.7	1.724	1.93	2.29
pk12-MA::Art v 1-His (C26W) 2	100.6	0.503	1.91	2.37
pk12-MA::Art v 1-His (E28R) 1	133.6	0.668	1.90	2.33
pk12-MA::Art v 1-His (E28R) 2	177.7	0.889	1.92	2.32
pk12-mIL-15-GPI 1	198.5	0.993	1.93	2.33
pk12-mIL-15-GPI 2	157.8	0.789	1.91	2.32
pk12-MA::Art v 1-His 1	269.7	1.349	1.93	2.3
pk12-MA::Art v 1-His 2	328.9	1.645	1.93	2.28
pk12-MA::Art v 1-His 3	341.4	1.707	1.90	2.24
pk12-MA::Art v 1-His 4	410.2	2.051	1.93	2.28

Although higher results of the 260/280 ratio do not indicate any issues regarding the purity of the sample, the pk12-MA::Art v 1-His (C26S 1) plasmid revealed a value of 2.04, which could be reasoned with an RNA contamination. The gathered data of the 260/230 ratio displayed values between 2.24–2.41, excluding possible reasons for higher results, like inappropriate solution for the blank measurement and a dirty pedestal, the measurements are acceptably higher than 2.2. (Assessment of Nucleic Acid Purity 2011) In summary, the purity of the obtained plasmids and exclusion of protein, RNA or any other kind of contamination was confirmed.

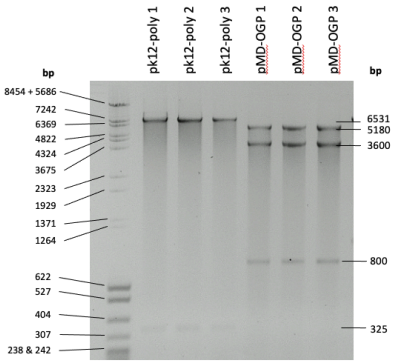
3.2 Verification of extracted plasmid DNA with restriction digestion

After the DNA extraction, the transformed his-tagged plasmid DNA was digested with XhoI and NotI-HF restriction enzymes and verified for its size with an agarose gel electrophoresis run. All the samples were confirmed for their correct vector and insert sizes. The expected and verified size of the DNA samples are listed in **Table 7** and the corresponding pictures of the gel electrophoresis runs are displayed in **Figure 3**.

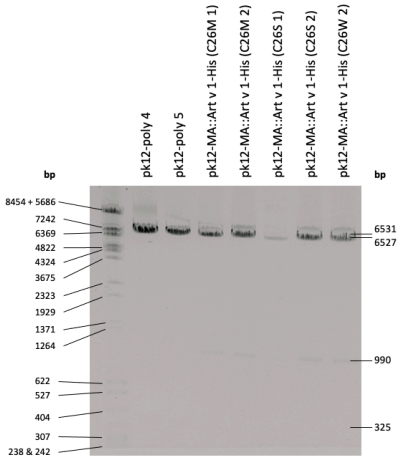
Table 7: List of the expected and confirmed vector and insert sizes in base pairs (bp) of the obtained plasmid DNA samples.

Plasmid sample	Vector size [bp]	Insert size [bp]
pk12-poly	6531	325
pMD-OGP	5180	3600 & 800
pk12-MA::Art v 1-His (C26M)	6527	990
pk12-MA::Art v 1-His (C26S)	6527	990
pk12-MA::Art v 1-His (C26W)	6527	990
pk12-MA::Art v 1-His (E28R)	6527	990
pk12-MA::Art v 1-His	6527	990
pk12-mIL-15-GPI	6531	873

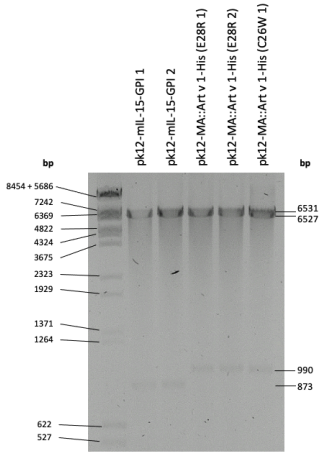
(A)



(B)



(C)



(D)

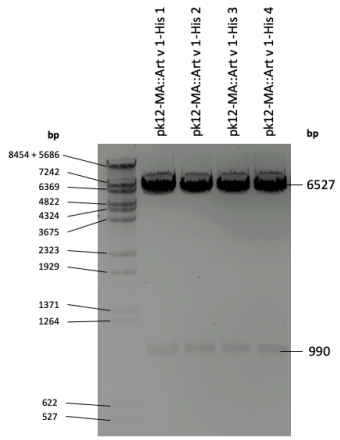


Figure 3: Verification of obtained plasmid DNA through restriction digestion with XhoI and NotI-HF restriction enzymes in CutSmart-buffer, followed by 1 % agarose gel electrophoresis with 1x loading dye.
For the determination of the sizes, the pBR322 DNA-MspI Digest (size range: 117 bp–8,454 bp) and the λ DNA-BstEII Digest (size range: 9 bp–622 bp) markers were used. (A) Shows pk12-poly samples 1–3 with vector size of 6531 bp and an insert of 325 bp and also pMD-OGP samples 1–3 with sizes of 5180 bp, 3600 bp and 800 bp. (B) Confirms the vector and insert sizes of pk12-poly 4–5, MA::Art v 1-His (C26M 1–2), pk12-MA::Art v 1-His (C26S 1–2) and pk12-MA::Art v 1-His (C26W 2). The pk12-poly samples displayed bands at 6531 bp and 325 bp. The sizes of the MA::Art v 1-His APLs were confirmed with vector sizes of 6527 bp and the inserts with 900 bp each. (C) Displays the verified cytokine (pk12-mIL-15-GPI) plasmid size with 6531 bp of the vector and 873 bp of the insert as well as 6527 bp with inserts of 990 bp for the other APLs (E28R 1–2 and C26W 1). (D) The MA::Art v 1-His tagged plasmid DNAs 1–4 were verified with vector sizes of 6527 bp and inserts of 990 bp.

3.3 Verification of extracted plasmid DNA with DNA sequencing

For verification, the sequenced plasmid DNAs were aligned to the MA::Art v 1-His control sequence. The alignments are displayed in **Figure 4**, and the his-tags (black), as well as the substitutions (red, green), are marked. The alignments show confirmation of the 6x His-tag (CATCACCATCATCATCAT) located at the C-terminal of Art v 1 in all sequences and the respective amino acid substitutions at position 26 or 28 in the altered his-tagged MA::Art v 1 sequences (C26M, C26S, C26W, E28R). **Table 8** displays the mutation sites, the exchanged amino acids and their corresponding codons and the corresponding Figure they are displayed in.

Table 8: Overview of the amino acid substitutions of the different APLs.
Shown are the plasmid, the position and original amino acid in the translated protein and the corresponding codon as well as the altered amino acid with its codon and the Figure in which the alignments are displayed.

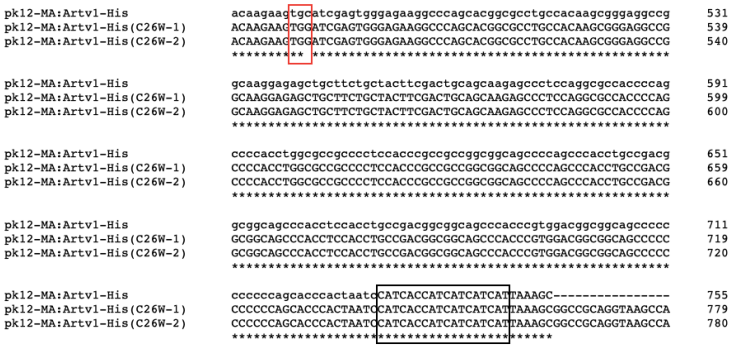
Plasmid	Amino acid position	Original Amino Acid	Codon for original amino acid	Altered amino acid	Codon for altered amino acid	Figure
pk12-MA::Art v 1 (C26M)	26	Cysteine	TGC	Methionine	ATG	Figure 4A
pk12-MA::Art v 1 (C26S)	26	Cysteine	TGC	Serine	AGC	Figure 4B

pk12-MA::Art v 1 (C26W)	26	Cysteine	TGC	Tryptophan	TGG	Figure 4C
pk12-MA::Art v 1 (E28R)	28	Glutamic acid	GAG	Arginin	AGG	Figure 4D

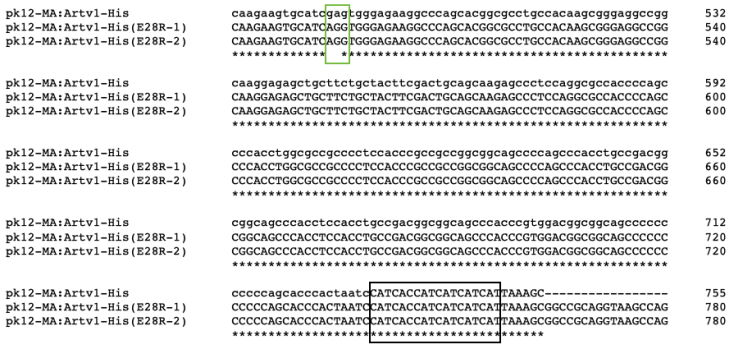
(A)

(B)

(C)



(D)



(E)

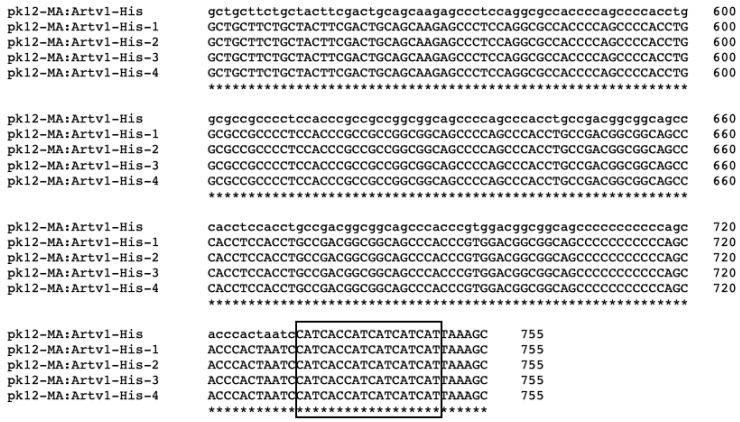


Figure 4: Multiple Alignments of the obtained plasmid DNAs after DNA extraction from E. coli cells with the caesium chloride density-gradient method in comparison with the wild-type his tagged control sequence of pk12-MA::Art v 1 -His. The red boxes mark the amino acid substitution sites at position 26 (A; B, C), the green one the substitution site at position 28 (D) and the black boxes indicate the his-tag in each sequence. (A) Shows the sequences of C26M-1 and -2 with the control sequence. At amino acid position 26 in the APL sequences, cysteine is exchanged with methionine. (B) Shows the sequences of C26S-1 and -2 with the control sequence. At amino acid position 26 in the APL sequences, cysteine is exchanged with serine. (C) Shows the sequences of C26W-1 and -2 with the control sequence. At amino acid position 26 in the APL sequences, cysteine is exchanged with tryptophan. (D) Shows the sequences of E28R-1 and -2 with the control sequence. At amino acid position 28 in the APL sequences, glutamic acid is exchanged with arginine. (E) Shows obtained his-tagged MA::Art v 1 sequences with complete identity to the control sequence.

3.4 Intracellular and extracellular flow cytometry staining

In a next step, the accuracy of expression of the different constructs was tested. For that purpose, the transfected H293T cells were stained intracellularly for the measurement of the his-tagged Art v 1 forms as well as extracellularly for the detection of mIL-15. The cell populations were gated according to their FSC area and height, exclusion of doublets followed by gating on live and un-damaged cells with the measurement of the mean fluorescence

intensity (MFI) of Zombie Aqua™ Dye-stained cells in the KO525-A channel (**Figure 5A**). With the anti-his and anti-Art v 1 staining, the MFI of the APL and pk12-MA::Art v 1-His WT transfected cells were compared to cells transfected with the poly plasmid used as non-his containing control. For the extracellular staining the anti-mIL-15-stained cells were compared to cells which were stained only with the secondary antibody. Representative graphs indicating the respective fluorescence intensities are shown in Figure 5**C** and **D** and confirm that all the transfected different plasmids showed proper cellular expression.

The anti-mIL-15-stained cells showed proper expression with an MFI of 96163 in comparison to the cells, which were stained only with the secondary antibody, showing an MFI of 7247 instead.

The anti-his staining features lower mean MFI of all the expressed plasmids in the first and third experiments with 18694 and 17284, in comparison to the second experiment with 35252. The anti-Art v 1 staining shows a lower mean MFI of all the expressed plasmids in the second experiment with 74446 than in the first and third experiments with 147936 and 141734. Regarding the different plasmids, the mean MFI of each staining is displayed in **Table 9.**

In conclusion of the mean expressions in each staining, pk12-MA::Art v 1-His was highest in the anti-Art v 1 staining but lowest in the anti-his staining. The pk12-MA::Art v 1-His (C26W) showed one of the higher expressions in the ant-his staining but lowest in the anti-Art v 1 staining.

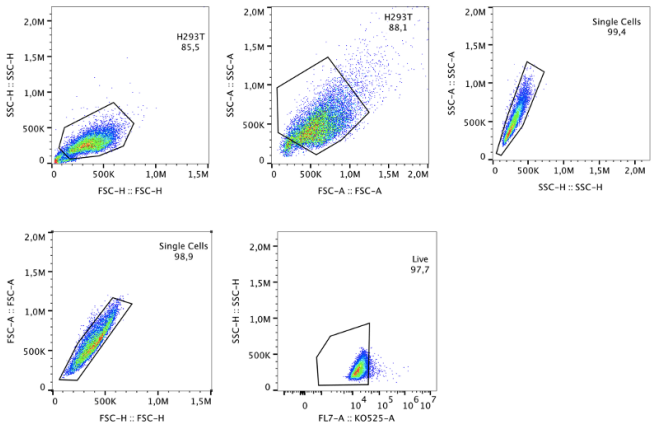
The statistical investigation was carried out with GraphPad Prism version 9.4.0 using a Kruskal-Wallis test for non-parametric data. Data did not show significant p-values and hence, confirms no significant variation in the expression of different APL in H293T cells.

Table 9: Mean MFI overview of APL and WT pk12-MA::Art v 1-His plasmids in transient transfected H293T cells stained against their his tag and Art v 1 for n = 3 experiments.

Plasmid	**Mean ± SD of anti-his staining**	**Mean ± SD of anti-Art v 1 staining**
pk12-MA::Art v 1-His (C26M)	23734 ± 5234	96399 ± 43757
pk12-MA::Art v 1-His (C26S)	27802 ± 9767	109314 ± 34377

pk12-MA::Art v 1-His (C26W)	27364 ± 16317	71001 ± 50653
pk12-MA::Art v 1-His (E28R)	19540 ± 11639	99580 ± 68416
pk12-MA::Art v 1-His	17286 ± 4944	128670 ± 33219
pk12-poly	7016 ± 2227	35204 ± 13955

(A)



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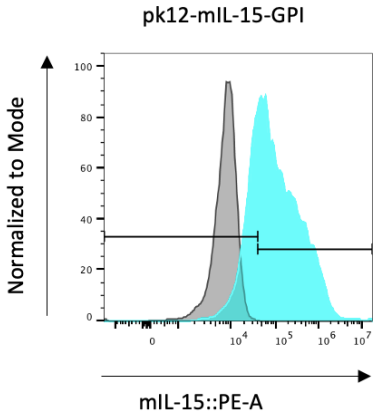

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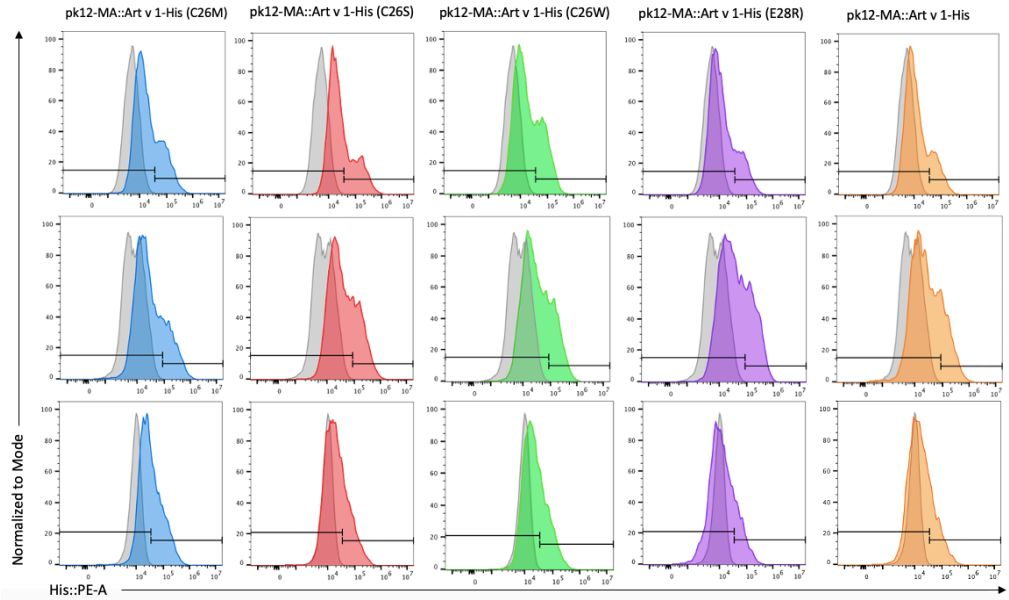

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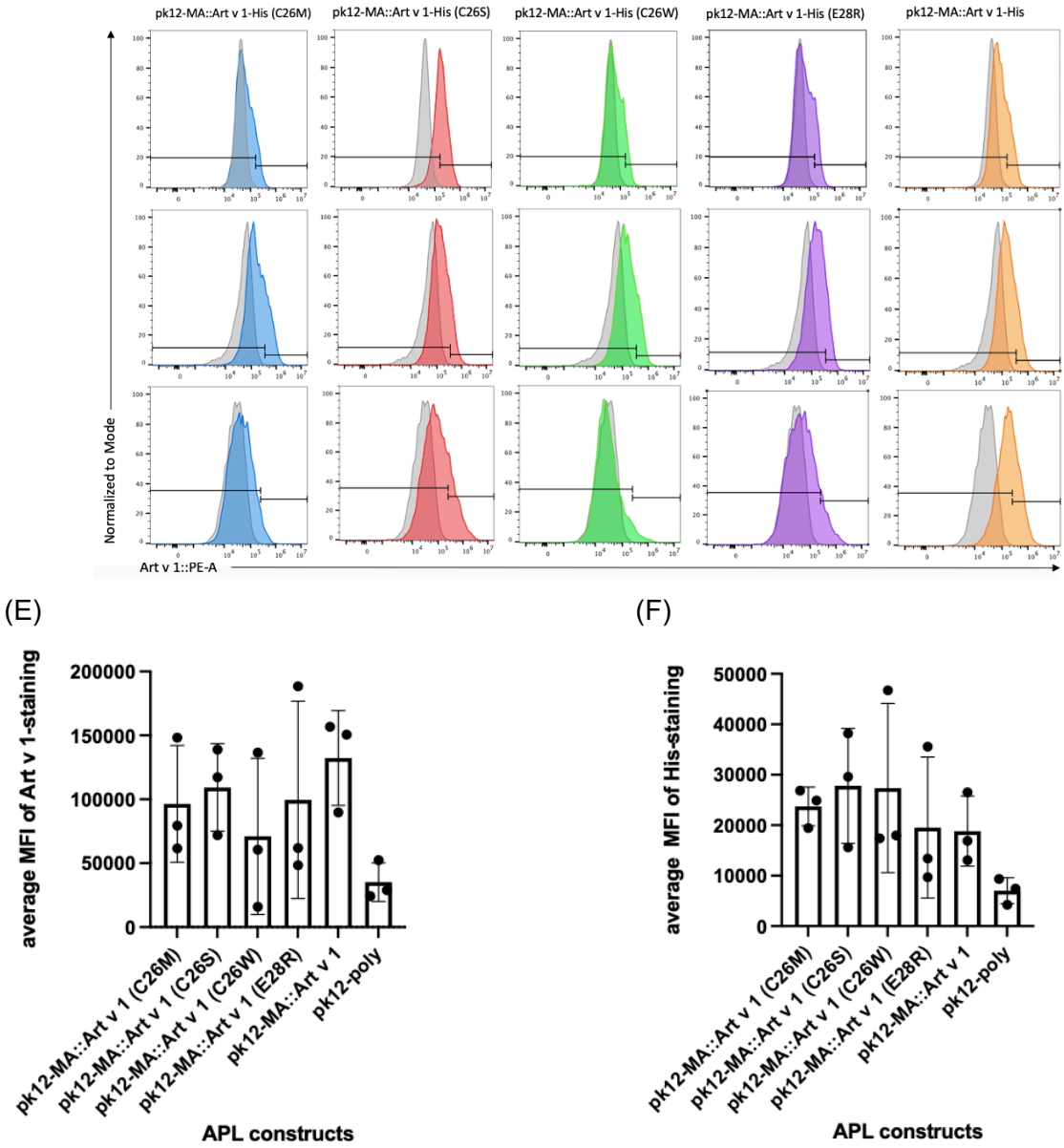

Figure 5: Determination of the proper plasmid expression of the obtained and verified plasmid DNAs with transient transfection of H293T cells with each plasmid.

(A) Shows the gating strategy of the transiently transfected cells in the FACS-staining. The gating was performed for the size of H293T cells, single cells and living cells, excluding dead cells due to dying with Zombie Aqua™ Dye. (B) Confirms proper expression of the transiently transfected cells with the pk12-mIL-15 plasmid, stained with mouse IL-15 antibody (polyclonal goat IgG) and phycoerythrin-conjugated AffiniPure F(ab')2 fragment donkey anti-goat IgG (H+L) and compared to a secondary antibody only-staining. (C) Anti-his staining of the plasmid-expressing H293T cells transiently transfected with the different APL plasmids (MA::Art v 1-His (C26M), MA::Art v 1-His (C26S), MA::Art v 1-His (C26W), MA::Art v 1-His (E28R), MA::Art v 1-His) against with pk12-poly transiently transfected cells. The his-tag was directly detected with a PE anti-His tag (Clone: J095G46, Isotype: Mouse IgG2a). (D) Displays the proper expression of anti-Art v 1 stained with the APL plasmids transfected H293T cells compared to cells transfected with pk12-poly plasmid DNA. Indirect staining with rabbit-anti-Art v 1 peptide 4 anti-serum and

PE anti-rabbit IgG (phycoerythrin-conjugated affinipure F(ab')2 fragment goat anti-rabbit IgG (H+L)) was carried out. (E) Shows the comparison of the mean and SD of the different expressions of the APL plasmids (pk12-MA::Art v 1-His (C26M), pk12-MA::Art v 1-His (C26S), pk12-MA::Art v 1-His (C26W), pk12-MA::Art v 1-His (E28R), pk12-MA::Art v 1-His) against pk12-poly from the anti-Art v 1 staining. (F) shows the comparison of the mean and SD of the different expressions of the APL plasmids (pk12-MA::Art v 1-His (C26M), pk12-MA::Art v 1-His (C26S), pk12-MA::Art v 1-His (C26W), pk12-MA::Art v 1-His (E28R), pk12-MA::Art v 1-His) against pk12-poly from the anti-his staining. Statistical testing was performed with a Wilcoxon Rank sum test for non-parametric data for testing the significant differences between the different APL plasmids. No significant p-values were reported and hence not shown in the graphs (E, F).

3.5 Micro BCA Protein Assay

The total protein amount of the different VNP preparations was determined with the Micro BCA Protein Assay with BSA standard concentrations from 0.5–200 µg/ml and measurements at 562 nm for examination of the VNP expression in the H293T cells. **Table 10** displays the protein concentrations of VNPs obtained from each transfected culture dish, their mean concentrations, the total volume, and the total protein amount of the obtained VNPs in relation to the volume**. Figure 6** shows a comparison of the total obtained protein amount of each VNP preparation. The total protein amounts ranged from ~270–990 µg with poly VNP and poly + mIL-15 VNP showing the highest levels of protein. Regarding the APL VNP, WT + mIL-15 VNP displayed the highest amounts with ~610 µg and C26M + mIL-15 the lowest with a total protein amount of 270 µg.

Table 10: Results of the Micro BCA Protein Assay of the different VNP preparations. and characterized for their total protein amount in µg/ml.
The samples were measured at 562 nm and the concentrations were calculated in relation to the used BSA standard concentrations ranging from 0.5–200 µg/ml. The table gives an overview of the concentration measurements of each transfected dish in µg/ml, the mean of the three concentrations in µg/ml, the total volume in µl and the calculation for the obtained total protein amount in µg.

VNP preparations	Protein concentration/plate [µg/ml]	Mean Concentration [µg/ml]	Volume [µl]	total protein [µg]
poly VNP	2135,83	2481,61	400	992,6
	2587,67			
	2721,33			
WT VNP	1180,50	1342,74	400	537,1

	1366,67 1481,07			
WT + mIL-15 VNP	1401,50 1576,00 1621,20	1532,90	400	613,2
C26M VNP	1385,00 1386,33 1656,00	1475,78	200	295,2
C26M + mIL-15 VNP	1299,83 1319,00 1437,07	1351,97	200	270,4
C26S VNP	1692,50 1802,00 2062,13	1852,21	200	370,4
C26S + mIL-15 VNP	1666,83 1748,33 1851,20	1755,46	200	351,1
C26W VNP	1690,00 1600,00 1737,60	1675,87	200	335,2
C26W + mIL-15 VNP	1673,83 1773,33 1939,20	1795,46	200	359,1
E28R VNP	898,50 1041,47 1043,20	994,39	400	397,8
E28R + mIL-15 VNP	1150,00 1172,00 1250,80	1190,93	400	476,4
poly + mIL-15 VNP	1931,50 2256,33 2294,67	2160,83	400	864,3

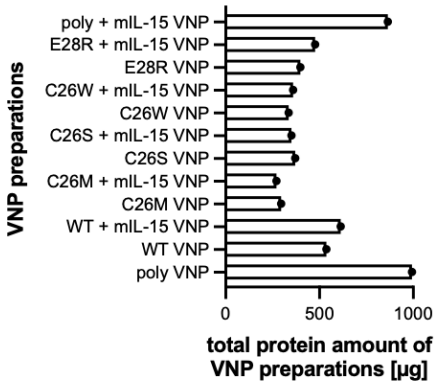

Figure 6: Comparison of the obtained total protein amount of the VNP preparations listed in Table 10.

3.6 ELISA

With the double sandwich ELISA, the percentage of Art v 1 inside the VNP preparations was determined in relation to the WT + mIL-15 VNP containing VNPs. **Table 11** shows the mean absorbance (Abs) of the measured technical triplicates of each applied concentration (3, 0.3 and 0.1 µg/ml), already adjusted the Abs of the different concentrations to 1 µg/ml as well as the mean Abs of the VNP preparations minus the background Abs corresponding to the poly VNP and the percentage of Art v 1 in the VNPs in comparison to the WT + mIL-15 VNP. The percentages are displayed in **Figure 7** and range between 74 % and 145 %.

Table 11: Absorbance (Abs) measurements of the double sandwich ELISA for determination of the percentage of Art v 1 inside the VNP preparations.
The table shows the mean Abs of the resulting measurements, the mean Abs after subtracting the Abs of poly VNP and the percentage of Art v 1 in the VNP preparations in relation to the WT + mIL-15 VNP.

VNP preparations	Mean Abs of all replicates & conc.	Mean Abs – poly VNP Abs	Percentage of Art v 1 in VNPs compared to WT + mIL-15 VNP
poly VNP	0,55		
WT VNP	1,23	0,68	109%
WT + mIL-15 VNP	**1,17**	**0,62**	**100%**
C26M VNP	1,32	0,77	124%
C26M + mIL-15 VNP	1,45	0,90	145%
C26S VNP	1,16	0,61	98%
C26S + mIL-15 VNP	1,01	0,46	74%
C26W VNP	1,21	0,67	107%
C26W + mIL-15 VNP	1,27	0,72	116%
E28R VNP	1,13	0,58	94%
E28R + mIL-15 VNP	1,05	0,50	81%

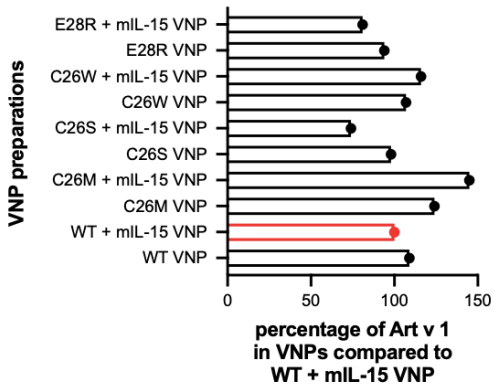

Figure 7: Comparison of the percentages of Art v 1 in the different VNP preparations in relation to pk12-MA::Art v 1 + pk12-mIL-15-GPI

3.7 Western Blot

With the western blot, the size and composition of the different VNP preparations were confirmed. For that purpose, the VNP preparations were applied on an SDS PAGE with a 4–18 % gradient gel, blotted onto a PVDF membrane, stained with mouse anti-his IgG antibody followed by ECL HRP-conjugated sheep anti-mouse whole antibody IgG and measured with Clarity™ Western ECL Substrate in the Fuji Luminescence Image Analyzer. The VNP preparations (poly VNP, WT VNP, WT + mIL-15 VNP, C26M VNP, C26M + mIL-15 VNP, C26S VNP, C26S + mIL-15 VNP, C26W VNP, C26W + mIL-15 VNP, E28R VNP, E28R + mIL-15 VNP, poly + mIL-15 VNP) were applied together with a PageRuler Plus Prestained Protein Ladder (10-250 kDa). The sizes of the VNP preparations with ~26 kDa and the p30gag protein with 30 kDa display the according sizes in relation to the protein ladder. WT+ mIL-15 VNP and C26S + mIL-15 VNP showed low and C26W + mIL-15 VNP, E28R VNP and E28R + mIL-15 VNP high intensity of the bands in comparison between the different VNP preparations. The anti-his and p30gag stained membrane is shown in **Figure 8.**

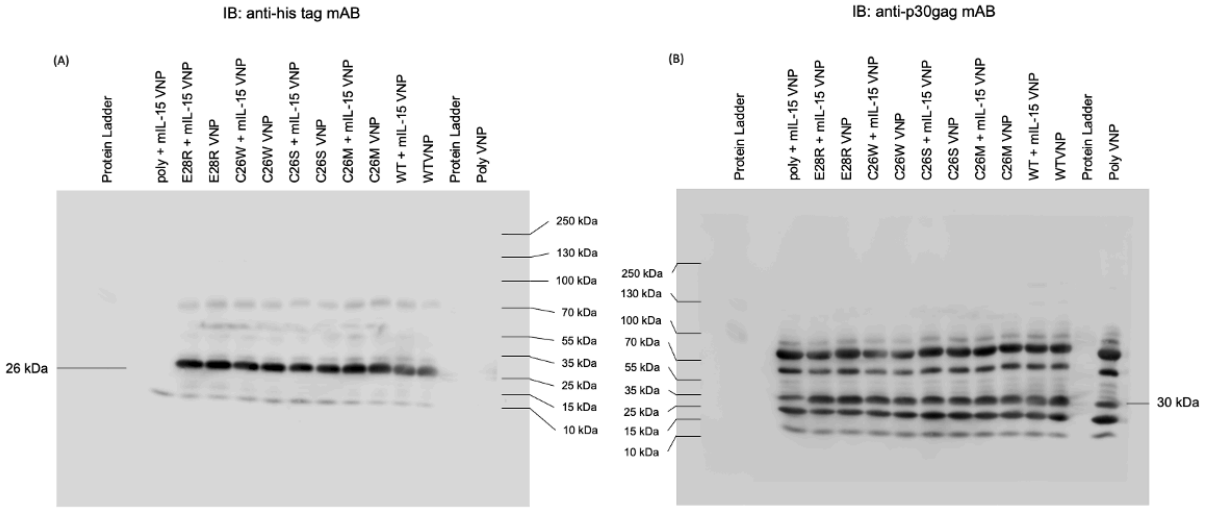

Figure 8: Characterization by Western Blot of the VNP preparations stained for the His-Tag (A) or p30gag (B). PageRuler Plus Prestained Protein Ladder (10-250 kDa) and the VNP preparations (poly VNP, WT VNP, WT + mIL-15 VNP, C26M VNP, C26M + mIL-15 VNP, C26S VNP, C26S + mIL-15 VNP, C26W VNP, C26W + mIL-15 VNP, E28R VNP, E28R + mIL-15 VNP, poly + mIL-15 VNP) with 15 µg each were applied. (A) Displays the membrane stained against the His-tag with mouse anti-his IgG antibody (Clone 13/45/31, 1 mg/ml) followed by ECL HRP-conjugated sheep anti-mouse whole antibody IgG and measured with ClarityTM Western ECL Substrate in the Fuji Luminescence Image Analyzer. (B) Displays the membrane stained against the p30gag protein with anti-p30gag rat sera followed by ECL anti-rat IgG (Horseradish Peroxidase linked whole antibody (from goat)) and measured with ClarityTM Western ECL Substrate in the Fuji Luminescence Image Analyzer.

3.8 Rat basophil leukemia cell-based immunological assay

Subsequently, we were interested to determine the potential allergenicity of the obtained VNP preparations containing the different forms of the Art v 1 protein. This was important to consider, since only hypoallergenic VNP preparations can be applied savely to patients. The hypoallergenicity of the different VNP preparations was determined with a rat basophil leukemia cell-based immunological assay by measuring the release of β-hexosaminidase with the different VNP preparations stimulated RBL cells. The percentage of release was ascertained in relation to 100 % release of the cells administrated with 1 % Triton X-100 and is displayed in **Figure 9** and the corresponding measurements are listed in **Table 12.** The positive control was 10 µg/ml anti-mouse IgE monoclonal cross-linking antibody (clone: R35-72) while rArt v 1 was used in concentrations of 0.01, 0.1, 1 and 10 µg/ml. The WT VNP, WT + mIL-15 VNP, C26M + mIL-15 VNP and E28R + mIL-15 VNP stimulated an increased β-hexosaminidase release only at higher concentrations (10 and 100 µg/ml), whereas E28R VNP induced higher release at all concentrations with the highest levels at 0.1 and 100 µg/ml. C26M VNP, C26S + mIL-15 VNP, C26W VNP, C26W + mIL-15 VNP, similar to empty VNPs (poly VNP), remained hypoallergenic at all concentrations tested. Poly + mIL-15 VNP induced a higher release at the lowest concentration (0.1 µg/ml).

Table 12: Results of the hypoallergenicity testing with a rat basophil leukemia immunological assay.
It shows percentages of release of β-hexosaminidase from Art v 1 sensitized RBL-2H3 cells after stimulation with different VNP preparations in relation to 1 % Triton X-100 (representing 100 % release). Recombinant Art v 1 in concentrations of 0.01, 0.1, 1 and 10 µg/ml and anti-IgE with 10 µg/ml as control were added on each plate (A, B, C). (A) Shows the measurements of the VNP preparations poly VNP, WT VNP, WT + mIL-15 VNP, C26M VNP and C26M + mIL-15 VNP in concentrations of 0.1, 1, 10 and 100 µg/ml of the total protein amount. (B) Displays the measurements of the VNP preparations C26S VNP, C26S + mIL-15 VNP, C26W VNP, C26W + mIL-15 VNP and E28R VNP in concentrations of 0.1, 1, 10 and 100 µg/ml of the total protein amount. (C) Shows the measurements of the VNP preparations E28R + mIL-15 VNP and poly + mIL-15 VNP, in concentrations of 0.1, 1, 10 and 100 µg/ml of the total protein amount.

(A)

	% of maximum release of β-hexosaminidase at:				
VNP preparation	**0.01 µg/ml**	**0.1 µg/ml**	**1 µg/ml**	**10 µg/ml**	**100 µg/ml**
rArt v 1	17.9	17.7	18.4	19.2	-
Anti-IgE	-	-	-	35.0	-
poly VNP	-	12.3	10.6	11.2	11.1

WT VNP	-	12.3	12.4	14.9	20.2
WT + mIL-15 VNP	-	12.3	14.4	17.2	28.1
C26M VNP	-	12.4	10.9	10.7	13.4
C26M + mIL15 VNP	-	14.1	12.6	14.0	27.7

(B)

	% of maximum release of β-hexosaminidase at:				
VNP preparation	**0.01 µg/ml**	**0.1 µg/ml**	**1 µg/ml**	**10 µg/ml**	**100 µg/ml**
rArt v 1	19,9	19,9	21,7	23,6	-
anti-IgE	-	-	-	34,0	-
C26S VNP	-	13,8	12,2	12,2	15,3
C26S + mIL15 VNP	-	14,6	12,1	12,4	16,8
C26W VNP	-	14,7	11,5	11,7	13,4
C26W + mIL15 VNP	-	16,9	13,2	12,9	14,5
E28R VNP	-	29,0	17,5	20,3	26,1

(C)

	% of maximum release of β-hexosaminidase at:				
VNP preparation	**0.01 µg/ml**	**0.1 µg/ml**	**1 µg/ml**	**10 µg/ml**	**100 µg/ml**
rArt v 1	22,3	23,0	24,3	24,2	-
anti-IgE	-	-	-	40,0	-
E28R + mIL-15 VNP	-	20,5	15,3	19,1	26,2
poly + mIL-15 VNP	-	25,0	15,1	14,1	15,8

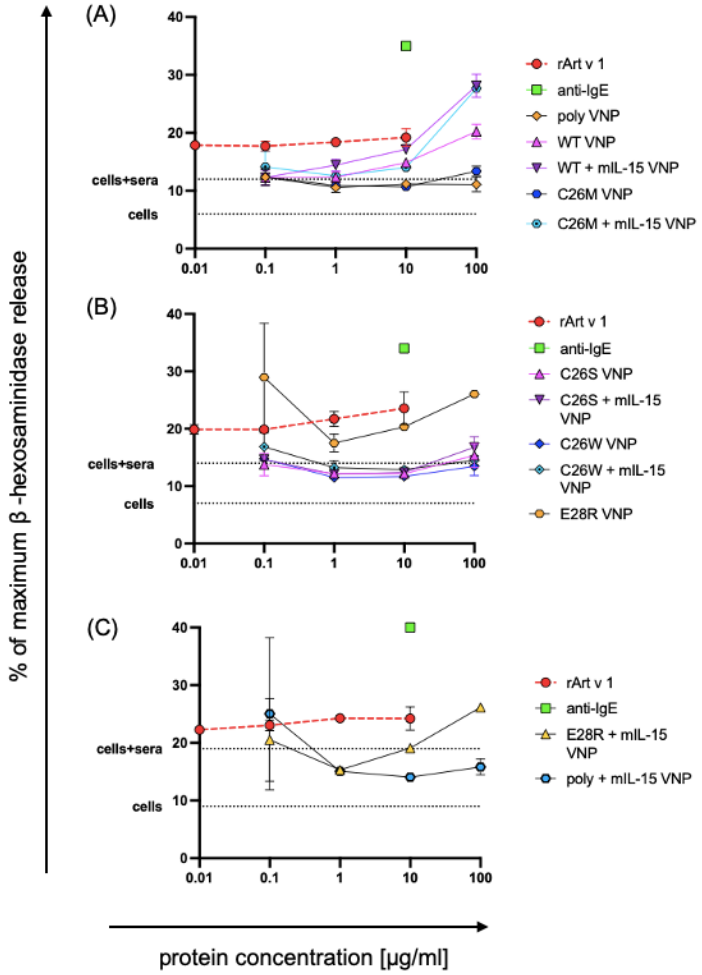

Figure 9: Results of hypoallergenicity testing with a rat basophil leukemia immunological assay.
Art v 1 sensitized RBL-2H3 cells were stimulated with 10 µg/ml cross-linking anti-IgE as positive control, rArt v 1 in concentrations of 0.01, 0.1, 1 and 10 µg/ml and 1 % Triton X-100, VNP preparations in concentrations of 0.1, 1, 10 and 100 µg/ml and the negative controls (Tyrode/BSA buffer with only cells and cells+sera) and measured for their β-hexosaminidase release at 465 nm. The percentage was determined in relation to the measurements of 1 % Triton X-100 (representing 100 % release). (A) Shows poly VNP, WT VNP, WT + mIL-15 VNP, C26M VNP, C26M + mIL-15 VNP compared to the anti-IgE and rArt v 1 controls and a threshold of only cells and only cells+sera as negative controls. (B) Displays C26S VNP, C26S + mIL-15 VNP, C26W VNP, C26W + mIL-15 VNP, E28R VNP compared to the anti-IgE and rArt v 1 controls and a threshold of only cells and only cells+sera as negative controls. (C) Shows E28R + mIL-15 VNP, poly + mIL-15 VNP compared to the anti-IgE and rArt v 1 controls and a threshold of only cells and only cells+sera as negative controls.

4. Discussion

In the process of this thesis, VNP shielding full-length mutated Art v 1 with membrane-bound murine IL-15 for immunomodulation of allergen-specific T cells could be successfully produced, characterized and their hypoallergenicity evaluated.

To test the sequence-confirmed expression plasmids, the HEK293T cells were transiently transfected with the obtained plasmid DNA for examination of their proper cellular expression before VNP production. Due to the linkage of MA::Art v 1 to the inner leaflet of the VNP, the plasmids pk12-MA::Art v 1, pk12-MA::Art v 1 (C26M), pk12-MA::Art v 1 (C26S), pk12-MA::Art v 1 (C26W), pk12-MA::Art v 1 (E28R) and pk12-poly were probed by intracellular staining while the putatively surface-expressed pk12-mIL-15-GPI was stained by standard surface staining of producer cells.

The results of the mIL-15 staining showed proper surface expression of IL-15. Though the experiment was performed only once, repetition of such an experiment can further validate the finding. In addition, such staining could also be performed on H293T cells transfected with both pk12-mIL-15-GPI as well as pk12-MA::Art v 1-his plasmids to reveal that the producer cell line can express the allergen inside and the cytokine on the surface.

The considerable variation between the two intracellular staining exeriments may have been caused by the general differences in expression levels observed between the different experiments performed. The differences between the repetition experiments may have been caused by variations in the cell density of the transduced H293T cells in the plates before transfection, or, alternatively, the varying binding strength of the antibodies used in the staining for the different APL. In general, the anti-his stainings showed less variation in the expression levels of the constructs tested than the Art v 1-specific stainings. The differences may point towards a greater affinity of the rabbit anti-Art v 1-P4 anti-serum to the different APL, although the mutations in the N-terminal region of the molecule should not affect the recognition of the C terminal polyproline tail by the peptide 4 antiserum. In addition, the measurement of pk12-MA::Art v 1 (C26W) in the third experiment shows fluorescence intensity similar to pk12-poly. This could be due to ineffective permeabilization and/or fixation of the cells and hence improper targeting of the intracellular protein. The graphical analysis showed that all recombinant versions of Art v 1 were well-expressed inside and on the surface of H293T cells and therefore could be further used for subsequent VNP-production.

The statistical analysis did not show any significant difference in the cellular expression levels of pk12-MA::Art v 1, pk12-MA::Art v 1 (C26M), pk12-MA::Art v 1 (C26S), pk12-MA::Art v 1

(C26W) or pk12-MA::Art v 1 (E28R), when compared to each other, demonstrating the generally successful expression of APLs in the producer cell line. Although the experiments were performed three times, for further authentication of the results more biological replicates need to be carried out.

The determination of the total protein amounts of the VNP preparations revealed considerable but clearly different amounts of VNP produced when expressing different APLs. Although the strong differences between poly VNPs, with and without mIL-15, and the APL containing VNPs are not surprising, because the producer cell lines have to produce additional numbers of proteins (gag, pol, OGP, MA::Art v 1 APLs and eventually mIL-15-GPI) for the generation of the APL containing VNPs when compared to the generation of poly VNPs (gag, pol, OGP and eventually mIL-15-GPI), which finally may lead to less VNP production. The small differences between the total protein amounts of the APL containing VNPs can be explained by differences in the expression strength of constructs as already suspected by the observed variations in the FACS staining. Moreover, since the cytokine IL-15 is biologically active, this might also affect the production VNPs.
Subsequently, the double-sandwich ELISA was performed to further determine the actual percentage of Art v 1 of the total protein amount in the VNP preparations, which also showed clear differences between the different VNP preparations in relation to the WT + mIL-15 VNP. Interestingly, when we compared the results of the different VNP preparations obtained from within the BCA assays with those of the double-sandwich ELISA, there was no clear correlation observed between the concentrations of the VNP preparations and those of the Art v 1 constructs expressed. A comparison to rArt v 1-His standards would be necessary to properly quantify the absolute amounts of Art v 1 in the respective VNPs.

The results of the western blot could, however, validate the size and composition of the obtained VNP preparations. The bands showed different intensities, despite the same total protein amount loaded, which either may be due to incomplete loading of samples or it may reflect the variable degree of Art v 1 expression as already suspected in flow cytometry stainings. The additional bands which are visible in the p30gag immunoblot may represent precursor proteins of the Gag protein of different lengths, including the p30 protein and other gag proteins (p15, pp12 or p10), which have not been cleaved yet from the p65 precursor or have been further split from p30 proteins. In the anti-his immunoblot, the extra bands may represent non-specific binding of the primary or secondary antibodies, due to the high

concentration of the antibody, or cross-reactivity of the primary antibody with similar epitopes of histidine repeats in other gag or pol proteins.

Looking at the results of the RBL immunological assay, which was performed to examine the allergenicity of the VNP preparations, it shows that the WT VNP, WT + mIL-15 VNP, C26M + mIL-15 VNP and E28R + mIL-15 VNP stimulated an increased β-hexosaminidase release only at higher concentrations, whereas E28R VNP induced higher release at all concentrations with the highest levels at 0.1 and 100 µg/ml. Only the C26M VNP, C26S + mIL-15 VNP, C26W VNP, C26W + mIL-15 VNP, similar to empty VNPs (poly VNP), remained hypoallergenic at all concentrations tested. Because the RBL assay was carried out as the last experiments in a larger set of experiments performed within this thesis and about one month after the VNP production, we here cannot entirely exclude that the allergenicity of some of the VNP preparations may, at least in part, also be caused by degradation over time e.g., due to longer storage at room temperature during experimental handling instead of constant storage at 4 °C.

In summary, the VNP shielding full-length mutated Art v 1 and expressed along with membrane-bound murine IL-15 for immunomodulation of allergen-specific T cells were effectively produced and characterized. The hypoallergenicity for all VNPs could not be finally confirmed and therefore needs to be reproduced and retested. Within the ELISA experiments, rArt v 1-his standards need to be embedded and concentrations need to be established alongside for a realistic determination of the percentage of Art v 1 in the VNP preparations. Hence, the next step will be the testing of these VNP preparations *in vitro* and *in vivo* for immunomodulatory effects on allergen-specific Th cytokine profiles.

5. Summary

At least 95 % of mugwort allergic patients are sensitized against the major mugwort pollen allergen Art v 1, making it an interesting candidate for new treatments based on Allergen-specific immunotherapy (AIT). Hypoallergenic virus-like nanoparticles (VNP) encasing altered peptide ligands (APL) of Art v 1 and surface expressing murine IL-15 (mIL-15) are therefore being examined as a new approach to safely perform AIT. The APLs can cause differential T-cell receptor signals and surface-expressed mIL-15 also induces a shift towards Th1 cytokine production. HEK293T cells were transfected with three different plasmids, pMD-OGP (containing a gag-pol sequence of Moloney Murine Leukemia Virus (MoMLV)), pk12-MA::Art v 1 (Art v 1 fused to the MoMLV matrix protein, MAp15) and pk12-mIL-15-GPI (mIL-15 with a GPI-anchor attachment sequence, for additional Th1 priming) for VNP production. The proper cellular expression was checked with flow cytometric staining on transiently transfected HEK293T cells. The obtained VNPs were purified with successive rounds of ultracentrifugation washing steps. With the BCA protein assay, the total protein content of the VNPs and with a double-sandwich ELISA, the percentage of Art v 1 inside the VNPs were quantified in relation to the WT + mIL-15 VNP. Further biochemical characterization of the VNPs was performed through Western Blot with SDS-PAGE. The hypoallergenicity of the VNP preparation was tested using Art v 1-specific-IgE sensitized rat basophil leukemia cells as the readout. The VNPs shielded full-length his-tagged APLs of Art v 1 and expressed mIL-15 on their surface. The hypoallergenicity of some VNPs, the proper expression of the plasmids and the size of recombinant proteins were confirmed. In conclusion, the VNPs shielding the full-length his-tagged APLs of Art v 1 and expressing mIL-15 on their surface, were successfully produced and biochemically characterized. These new platforms can now be studied *in vitro* and *in vivo* for their immunomodulatory effects on allergen-specific Th cytokine production.

6. Zusammenfassung

Mindestens 95 % der Beifußallergiker sind gegen das Hauptallergen des Beifußpollen, Art v 1, sensibilisiert, was es zu einem interessanten Kandidaten für neue Behandlungen im Rahmen allergenspezifischer Immuntherapie (AIT) macht. Hypoallergene virusähnliche Nanopartikel (VNP), die modifizierte Peptidliganden (APL) von Art v 1 beinhalten und murines IL-15 (mIL-15) auf der Oberfläche exprimieren, werden in diesem Zusammenhang als neuer Ansatz für eine sichere Durchführung von AIT als Therapie oder Prävention der Beifußallergie untersucht. Die APL können unterschiedliche T-Zell-Rezeptor-Signale stimulieren und Oberflächen-exprimiertes mIL-15 induziert einen Wechsel zur Produktion von Th1-Zytokinen. Für die VNP-Produktion wurden HEK293T-Zellen mit drei verschiedenen Plasmiden transfiziert: pMD-OGP (enthält die gag-pol-Sequenz des Moloney Murine Leukemia Virus (MoMLV)), pk12-MA::Art v 1 (Art v 1 fusioniert mit dem MoMLV-Matrixprotein, MAp15) und pk12-mIL-15-GPI (mIL-15 mit einer GPI-Verankerungssequenz für zusätzliches Th1-Priming). Die zelluläre Expression wurde mit Immunhistochemie und Durchflusszytometrie in vorrübergehend transfizierten HEK293T-Zellen überprüft. Die erhaltenen VNPs wurden durch wiederholte Runden Ultrazentrifugation aufgereinigt. Mit dem BCA-Protein-Assay wurde der Gesamtproteingehalt der VNPs und mit einem “double-sandwich“ ELISA der Prozentsatz an Art v 1 in den VNPs in Relation zu den WT + mIL-15 VNP quantifiziert. Außerdem wurde ein Western Blot mit SDS-PAGE als weitere biochemische Charakterisierung der VNPs durchgeführt. Die Allergenität der VNP-Präparate wurde mit Art v 1-spezifischen IgE-sensibilisierten Ratten-Basophilen-Leukämiezellen getestet. Die VNP beinhalteten die APL des His-markierten Art v 1 und exprimierten mIL-15 auf ihrer Oberfläche. Die Hypoallergenität einiger VNP sowie die korrekte Expression der Plasmide und Größe der rekombinanten Proteine wurde bestätigt. Zusammenfassend lässt sich sagen, dass VNPs, welche Art v 1 in voller Länge beinhalten und mIL-15 auf ihrer Oberfläche exprimieren, erfolgreich hergestellt und biochemisch charakterisiert wurden. Diese neuen Plattformen können nun *in vitro* und *in vivo* auf ihre immunmodulatorische Wirkung auf die allergenspezifische Th-Zytokin-Produktion untersucht werden.

7. List of Abbreviations

Abs	Absorbance
AIT	allergen-specific immunotherapy
APC	Antigen presenting cell
APL	Altered peptide ligand
APS	ammonium persulfate
Bp	basepairs
BSA	Bovine serum albumin
C26M	pk12-MA:Art v 1-His (C26M)
C26M + mIL-15 VNP	pk12-MA::Art v 1 (C26M) + pk12-mIL-15-GPI
C26M VNP	pk12-MA::Art v 1 (C26M)
C26S	pk12-MA:Art v 1-His (C26S)
C26S + mIL-15 VNP	pk12-MA::Art v 1 (C26S) + pk12-mIL-15-GPI
C26S VNP	pk12-MA::Art v 1 (C26S)
C26W	pk12-MA:Art v 1-His (C27W)
C26W + mIL-15 VNP	pk12-MA::Art v 1 (C26W) + pk12-mIL-15-GPI
C26W VNP	pk12-MA::Art v 1 (C26W)
CO2	carbon dioxide
DC	dendritic cells
E28R	pk12-MA:Art v 1-His (E28R)
E28R + mIL-15 VNP	pk12-MA::Art v 1 (E28R) + pk12-mIL-15-GPI
E28R VNP	pk12-MA::Art v 1 (E28R)
ECL	enhanced chemiluminescence
E. coli	Escherichia coli
EtBr	Ethidium bromide
FACS	Fluorescense Activated Cell Sorting
FBS	Fetal Bovine Serum
FcεRI	High-affinity IgE-receptor
FcεRII	Low-affinity IgE-receptor
H293T cells	Human embryonic kidney 293T cells
HBS	HEPES buffered Saline
HLA	human leukocyte antigen

HRP	horseradish peroxidase
IFN	Interferon
Ig	Immunglobulin
IL	Interleukin
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
mIL-15	murine Interleukin-15
MoMLV	Moloney murine leukemia virus
NK cells	natural killer cells
PBS	Phosphate buffered Saline
PBS^{++}	PBS with added Calcium and Magnesium
PBST	Phosphate buffered saline with 0.1 % Tween20
PE	Phycoerythrin
poly + mIL-15 VNP	pk12-poly + pk12-mIL-15-GPI
poly VNP	pk12-poly
PVDF	polyvinylidene difluoride
rArt v 1	recombinant Art v 1
Rpm	revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TAE-buffer	Tris-acetate-EDTA-buffer
TBST	Tris buffered saline with 0.1 % Tween20
TCR	T-cell receptor
TE-buffer	Tris-EDTA-buffer
TEMED	N,N,N′,N′-Tetramethylethylendiamin
TGF	transforming growth factor
Th1 cells	T helper 1 cells
Th17 cells	T helper 17 cells
Th2 cells	T helper 2 cells
TMB	Tetramethylbenzidine
Treg cells	regulatory T cells

UV	ultraviolet
VNP	Virus-like nanoparticle
WT	Wildtype
WT + mIL-15 VNP	pk12-MA::Art v 1 + pk12-mIL-15-GPI
WT VNP	pk12-MA::Art v 1

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