Vaccination with recombinant modified vaccinia virus Ankara prevents the onset of intestinal allergy in mice

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Keywords
allergy vaccine; food allergy; modified vaccinia virus Ankara; mouse allergy model.

Abstract
Background: Modified vaccinia virus Ankara (MVA)-encoding antigens are considered as safe vaccine candidates for various infectious diseases in humans. Here, we investigated the immune-modulating properties of MVA-encoding ovalbumin (MVA-OVA) on the allergen-specific immune response.

Methods: The immune-modulating properties of MVA-OVA were investigated using GM-CSF-differentiated BMDCs from C57BL/6 mice. OVA expression upon MVA-OVA infection of BMDCs was monitored. Activation and maturation markers on viable MVA-OVA-infected mDCs were analyzed by flow cytometry. Secretion of INF-γ, IL-2, and IL-10 was determined in a co-culture of BMDCs infected with wtMVA or MVA-OVA and OVA-specific OT-I CD8+ and OT-II CD4+ T cells. BALB/c mice were vaccinated with wtMVA, MVA-OVA, or PBS, sensitized to OVA/alum and challenged with a diet containing chicken egg white. OVA-specific IgE, IgG1, and IgG2a and cytokine secretion from mesenteric lymph node (MLN) cells were analyzed. Body weight, body temperature, food uptake, intestinal inflammation, and health condition of mice were monitored.

Results: Infection with wtMVA and MVA-OVA induced comparable activation of mDCs. MVA-OVA-infected BMDCs expressed OVA and induced enhanced INF-γ and IL-2 secretion from OVA-specific CD8+ T cells in comparison with OVA, wtMVA, or OVA plus wtMVA. Prophylactic vaccination with MVA-OVA significantly repressed OVA-specific IgE, whereas OVA-specific IgG2a was induced. MVA-OVA vaccination suppressed TH2 cytokine production in MLN cells and prevented the onset of allergic symptoms and inflammation in a mouse model of OVA-induced intestinal allergy.

Conclusion: Modified vaccinia virus Ankara-ovalbumin (MVA-OVA) vaccination induces a strong OVA-specific TH1-immune response, likely mediated by the induction of INF-γ and IgG2a. Finally, MVA-based vaccines need to be evaluated for their therapeutic potential in established allergy models.

Abbreviations
APC, antigen-presenting cell; BHK, baby hamster kidney; BMDC, bone marrow-derived dendritic cell; CI, confidence interval; GM-CSF, granulocyte macrophage colony-stimulating factor; HRP, horseradish peroxidase; i.p., intraperitoneal; IFN, interferon; MVA, modified vaccinia virus Ankara; mDC, myeloid dendritic cell; MLN, mesenteric lymph node; MOI, multiplicity of infection; OVA, ovalbumin; o/n, over night; pfu, plaque forming unit; p.i., postinfection; RBL, rat basophilic leukemia; TNF, tumor necrosis factor; TMB, tetramethyl benzidine; VACV, vaccinia virus; wt, wild type.
The prevalence of food allergy is constantly increasing in westernized countries with approximately 5% of children and 3–4% of adults affected (1). While avoidance of the offending food and emergency medication still remains the only approved intervention strategies (2), several new approaches for allergen-specific immunotherapy are currently being evaluated, including gene transfer vectors derived from adenoviruses (3), lentivirus (4), and poxvirus (5).

Modified vaccinia virus Ankara (MVA) is a highly attenuated poxvirus with important safety features including its inability to productively replicate in human cells (6, 7). Despite a block of virion assembly, viral protein expression is fully functional and allows unimpaired synthesis of recombinant proteins (8) and efficient presentation of these antigens to the immune system. Wild-type (wt) MVA has been used safely as a vaccine against smallpox in the 1970s vaccination campaigns where more than 120,000 subjects in Germany were vaccinated, including patients with allergy or skin disease, infants, and children. Recent clinical trials with wtMVA as well as recombinant MVA vaccine candidates confirmed the safety of MVA in humans (9–11). In line with this, recombinant MVA vaccines have been shown to induce protective immunity in tumor therapy (12) and various infectious diseases (13–16).

The immune reaction upon MVA infection is mainly characterized by a TH1-dominated response (17). It has been shown that MVA preferentially infects dendritic cells (18) inducing increased levels of type I interferon (19). Using a mouse-pox vaccination model, the early protective effect of MVA was shown to be dependent on CD8+ T cells (20). Recombinant MVA induces the secretion of IFN-γ and MVA- and antigen-specific IgG2a antibodies in vivo (17). In a previous study, we found that prophylactic vaccination with a recombinant MVA that carries the cDNA for the model allergen ovalbumin (OVA, Gal d 2 from chicken, Gallus domesticus) was able to prevent induction of OVA-specific IgE antibodies in BALB/c mice (5).

Here, we investigated the immune-modulating properties of MVA-OVA in vitro and applied MVA-OVA vaccination to a mouse model of intestinal allergy. We showed prevention of the onset of allergic symptoms after oral food challenge which was associated with a shift from a Th2- to a Th1- immune response, both in vivo and in vitro.

Material and methods
Preparation and characterization of wtMVA and MVA-encoding OVA (MVA-OVA)
Modified vaccinia virus Ankara-ovalbumin (MVA-OVA) was generated as reported by El-Gogo et al. (21). Amplification and characterization of recombinant MVA-encoding OVA in terms of purity and endotoxin depletion are described in Data S1.

Mice
See Data S1.

MVA-OVA infection of murine-derived dendritic cells
Generation, infection, and activation of BMDCs, and OVA expression in infected cells are described in Data S1. Briefly, BMDCs derived from C57BL/6 mice were infected with a MOI of 1 and 10 of MVA-OVA or wtMVA, respectively. OVA expression was analyzed by immunoblotting using an OVA-specific mouse IgG1 and anti-mouse IgG-HRP. Expression of CD40, CD86, and CD69 on viable wtMVA and MVA-OVA-infected BMDCs were analyzed by flow cytometry. BMDCs were incubated for 2 h with wtMVA or MVA-OVA at a MOI of 1 and then washed to remove unbound viruses. 24 h after infection cells were stained with Viability DyeFlour780, FITC-labeled anti-CD40, FITC-labeled anti-CD86 or PE-labeled anti-CD69 (eBioscience, San Diego, CA, USA) and fixed with 4% paraformaldehyde. Cells were gated on viability dye<sup>low</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>B220<sup>−</sup> myeloid dendritic cells (viable mDCs).

Co-culture of MVA-OVA-infected BMDCs and OVA-specific T cells
Bone marrow-derived dendritic cell (BMDCs) (5 × 10<sup>5</sup>) were incubated for 2 h with wtMVA or MVA-OVA at a MOI of 1, washed, and co-cultured with 8 × 10<sup>5</sup> MACS-sorted splenic OVA-specific CD8<sup>+</sup> OT-I or CD4<sup>+</sup> OT-II T cells. As controls, cells were cultured with LPS (10 μg/ml) or OVA (100 μg/ml, LPS-free, Seikagaku Corporation, Tokyo, Japan). Supernatants were analyzed by ELISA for IL-2 (24 h p.i.), IFN-γ and IL-10 secretion (72 h p.i.; BD Biosciences, San Diego, CA, USA).

Vaccination, sensitization, and challenge of BALB/c mice
BALB/c mice (n = 10 treatment group, n = 5 sham group) were vaccinated twice (i.p.) in a 2-week interval with wtMVA (10<sup>7</sup> pfu), MVA-OVA (10<sup>7</sup> pfu) or PBS, respectively. Three weeks after the final vaccination, mice were sensitized (i.p.) with 50 μg OVA (Grade V, Sigma-Aldrich) adsorbed to 1 mg alum (Thermo Fisher, Rockford, IL, USA) twice at 2-weeks interval (22). One week after sensitization, mice were challenged for 6 days by feeding pellets that contained 20% chicken egg white (54% OVA content) or casein as control (ssniff GmbH, Soest, Germany; Fig. S2).

Determination of OVA-specific antibodies and monitoring disease symptoms
Ovalbumin (OVA)-specific IgE, IgG1, and IgG2a antibody levels were analyzed by ELISA using OVA-coated microtiter plates (50 μl/well, 100 μg/ml; Data S1). Presence of functional OVA-specific IgE in serum samples was analyzed by in vitro
mediator release assay using RBL 2H3 cells (23). Briefly, cells were incubated with mouse sera to allow IgE to bind to the FcεRI, washed, and stimulated with 0.1 mg/ml OVA (Grade V, Sigma-Aldrich). Secretion of β-hexosaminidase was measured. IgE blocking activity of sera from MVA-OVA-vaccinated mice was investigated by competitive ELISA (Data S1). Disease symptoms of mice were recorded according Schülke et al. (24). A symptom score from 0 to 5 was applied to assess the phenotype of mice. Roughness of fur and condition of feces were assessed in a blinded manner during challenge (roughness of fur: 0–2, condition of feces: 0–3, where 0 = hard feces, 1 = soft feces, 2 = liquid feces, and 3 = white, mucus like feces). Moreover, body weight, food uptake, and core body temperature were measured daily.

Cytokine secretion from mesenteric lymph node cells
Mesenteric lymph node (MLN) cells were prepared from OVA-sensitized and egg white diet fed BALB/c mice (allergic control, MVA, and MVA-OVA treated) using a cell strainer. Upon washing, MLN cells (4 × 10⁷/well) were cultured without restimulation for 72 h in RPMI 1640 (Invitrogen) supplemented with 5% FCS, 1000 U/l streptavidin-penicillin, 1.5 mg/l L-Glutamin, 7 µl/l β-mercaptoethanol. Supernatants were frozen at −20°C until determination of IL-4 and IFN-γ concentration by ELISA (BD OptEIA, BD Biosciences).

Histological analysis
See Data S1.

Statistical analysis
See Data S1.

Results
Bone marrow-derived DCs express OVA after infection with MVA-OVA
Ovalbumin (OVA) expression was highest in BMDCs infected with MVA-OVA at a MOI of 1 and was reduced at the higher MOI of 10 (Fig. 1), most probably due to infection-related induction of cell death which was observed in a dose-dependent manner at increased MOIs (data not shown). Upon co-incubation of BMDCs with wtMVA plus OVA, OVA was detected only in the supernatant but not in the cell lysate, indicating that infection with wtMVA did not facilitate uptake of exogenously added OVA (data not shown).

MVA-OVA infection activates dendritic cells and induces IFN-γ secretion from OVA-specific CD8⁺ T cells
Infection with both, wtMVA and MVA-OVA comparably activated and matured viable myeloid DCs (CD11b⁺ CD11c⁺ B220⁻) from C57BL/6 and BALB/c mice (data not shown) as indicated by up-regulation of CD40, CD86, and CD69 surface markers (Fig. 2). MVA-OVA-infected BMDCs induced a strong secretion of IL-2 (Fig. 3A) and IFN-γ (Fig. 3B) from OVA-specific CD8⁺ T cells, indicating substantial T cell activation. No secretion of IL-10 from infected BMDC or BMDC co-cultured T cells was detected (Fig. 3C). No cytokine secretion was detected after stimulation with either wtMVA, OVA or the mixture of wtMVA plus OVA. LPS induced IL-10 secretion by BMDCs, but no IFN-γ and IL-2 secretion by T cells. Of note, upon co-cultivation of MVA-OVA-infected BMDCs with OVA-specific OT-II CD4⁺ T-cells, no cytokine secretion was detected (Fig. 3D–F). In contrast, OVA-pulsed BMDC applied as positive control mediated the induction of IL-2 (Fig. 3D) and IFN-γ (Fig. 3E) from OVA-specific OT-II CD4⁺ T cells. Remarkably, secretion of both cytokines was suppressed by co-administration of MVA. In summary, MVA-OVA-infected antigen-presenting cells preferentially activate CD8⁺ T cells for secretion of both IL-2 and the Th1-promoting cytokine IFN-γ.

Vaccination of BALB/c mice with MVA-OVA prevents the induction of OVA-specific IgE and shifts the OVA-specific antibody response toward Th1
A mouse model of intestinal allergy (22) was applied to assess the efficacy of prophylactic MVA-OVA vaccination in vivo (Fig. S2). The time course of OVA-specific antibody response is depicted in Fig. S3. After challenge, OVA-specific IgE levels of MVA-OVA-vaccinated mice were significantly lower compared to the allergic controls (sensitized and challenged group) and to animals vaccinated with wtMVA (Fig. 4A). A significant suppression of functional OVA-specific IgE was confirmed by mediator release induced by sera from MVA-OVA-vaccinated mice (Fig. 4B). Measurement of OVA-specific IgG antibodies revealed differences in subtype distribution among the different treatment groups. While there was no significant difference between the IgG1 levels among the vaccinated and nonvaccinated mice (Fig. 4C), OVA-specific IgG2a antibody levels were significantly increased after challenge of mice vaccinated with MVA-OVA compared to the allergic group and wtMVA-vaccinated group (Fig. 4D). Consequently, prophylactic MVA-OVA
treatment significantly enhanced IgG2a/IgG1 and IgG2a/IgE ratios in comparison with the nonvaccinated allergic control group and wtMVA-treated group (Fig. 4E,F). Furthermore, sera from MVA-OVA-vaccinated mice were able to inhibit specific IgE-binding to OVA from patients with egg allergy and sensitization to OVA (data not shown). Noteworthy, IL-4 secretion from MLN cells derived from MVA-OVA vaccinated, and egg white diet challenged animals was reduced in comparison with allergic control mice, while IFN-γ secretion was increased (Fig. 5). Suppression of IL-4 by wtMVA vaccination was moderate, whereas IFN-γ induction was similar to the MVA-OVA-vaccinated group. In summary, prophylactic vaccination of mice with MVA-OVA was able to induce a systemic and local Th1 immune response upon oral challenge with OVA-containing food pellets.

Vaccination with MVA-OVA prevents the onset of clinical symptoms in a mouse model of intestinal allergy

Body weight and core body temperature after challenge were analyzed (Fig. 6A, B). While the sensitized and challenged allergic control group showed significant weight loss (mean: 7.05%, 95% CI: 9.38–4.72) and drop in core body temperature (mean: 5.82%, 95% CI: 7.06–4.58), symptoms were significantly decreased in MVA-OVA-treated mice (mean weight
loss: 0.93%, 95% CI: 3.25–1.40; mean core body temperature loss: 2.07%, 95% CI: 3.31–0.83). Furthermore, mean food uptake (g per mouse/day) of allergic controls challenged with egg white showed a decrease in food pellet uptake during the course of challenge (1.0–1.8 g/mouse/day), while uptake by MVA-OVA-vaccinated mice was comparable to values observed for both sensitized but not challenged and naïve mice (2.2–2.3 g/mouse/day vs 2.4–2.9 g/mouse/day; Fig. S4). The median symptom score in sensitized but nonvaccinated mice was three times higher than in MVA-OVA-vaccinated mice (median symptom score: allergic control: 1.5, MVA-OVA: 0.5, wtMVA: 2.0). Noteworthy, 5 of 10 MVA-OVA-vaccinated mice showed no symptoms at all (score = 0; Fig. 6C). Finally, histological analysis was performed to assess the efficacy of prophylactic MVA-OVA vaccination in allergic inflammation. Strong inflammatory changes, that is, goblet cell hyperplasia, crypt elongation, villous atrophy, and thickened basal layers, were observed in the small intestines of allergic control’s and MVA-treated mice, while only moderate goblet cell hyperplasia was observed in tissues of MVA-OVA-treated mice (Fig. 6D). Thus, prophylactic vaccination with MVA-OVA was able to prevent the onset of intestinal allergy, represses intestinal inflammation, and increases the production of OVA-specific IgG2a antibodies.

Discussion

The objective of our study was to evaluate the immune-modulating properties of MVA-encoding OVA (MVA-OVA). Former studies, using replication-deficient adenoviral vectors (3) as gene transfer vectors have shown to reduce allergic sensitization in mice. Nevertheless, adenoviruses promote an immune response against the viral vector, hampering repeated vaccinations (3, 25). In contrast, clinical trials indicated that MVA retains immunogenicity against the recombinant antigen upon multiple applications (26). MVA was found to be safe in humans (11) and can be applied as a shuttle vector to mediate allergen expression in infected host cells. In a previous proof-of-concept study, we demonstrated that vaccination with MVA-OVA protected mice from OVA-specific sensitization and induced a Th1-biased immune response (5). Here, we analyzed the capacity of MVA-OVA vaccination to prevent intestinal food allergy in mice. Furthermore, we investigated the immune stimulatory properties of MVA-OVA in vitro using murine BMDCs and OVA-specific T cells.

Modified vaccinia virus Ankara (MVA) preferentially targets immature professional APCs, in particular DCs and macrophages. In contrast to the nonattenuated vaccinia virus, MVA induces CD11c+DC maturation and IFN-α secretion (18). Because of the predominant capability for antigen presentation, we focused on mDCs differentiated from BM cells. Furthermore, only virus preparations...
characterized by high infectivity-to-MVA protein ratio have been used in this study. The rationale was to avoid noninfectious particles which potentially influence viral trafficking and might induce an undesired innate immune activation (27).

El-Gogo et al. (21) have shown that OVA is produced to high levels until 24 h postinfection when analyzing MVA-permissive BHK-21 cells infected with MVA-OVA at a MOI of 10. In our experiments with BMDCs, OVA levels peaked with infections at a MOI of 1 and were somewhat reduced upon infection with MOI of 10. This observation can be explained by the fact that high dose infections with MVA induce apoptosis of BMDCs (data not shown). In addition, upon low MOI infection of BMDC, bystander effects can lead to complete maturation of uninfected DCs (28). As wtMVA and MVA-OVA induce comparable activation and maturation of viable mDCs, we conclude that MVA-OVA retains the ability of wtMVA to activate and mature DCs.

Like other viruses, MVA induces a strong IFN-γ-mediated Th1 response. We showed MVA-OVA mediated IL-2 secretion as well as the induction of Th1 promoting cytokine IFN-γ could be attributed to OVA-specific CD8+ T cells. Remarkably, only MVA-OVA-infected BMDCs, but not wtMVA-infected BMDCs primed with LPS-free OVA, nor BMDCs primed with OVA alone induced IL-2 and IFN-γ secretion. This is in contrast to Nöder et al. (29), showing increased proliferation of CD8+ T cells co-cultured with BMDCs primed with a mixture of MVA plus OVA. Although identical mice strains were used the discrepancy might be explained by differences in the experimental setting in terms of stimulation protocols and OVA preparations. Depending on the experimental setting, OVA-specific CD8+ T cells are likely activated by direct and/or cross-presentation of antigens. MVA-infected DCs tend to undergo apoptosis and can subsequently be phagocytosed by noninfected DCs which may lead to bystander effects on T-cell activation (30, 31). Notably, cytokine secretion by OVA-specific CD4+ T cells could not be induced by MVA-OVA. Furthermore, OVA-induced cytokine secretion by CD4+ T cells is abolished by previous infection with wtMVA, indicating the immune-modulating properties of the virus. Results indicate a predominant MHC class I-restricted CD8+ T-cell response triggered by MVA-OVA infection. CD8+ T cells producing IFN-γ likely contribute to the mechanism by which MVA-OVA vaccination exerts its protective effect. This is in line with Tang et al. (32), who showed that CD8+ T cells can attenuate allergic inflammation in the lung of sensitized mice in an IFN-γ-dependent manner.

To investigate the disease and immune-modulating properties of MVA-OVA, we applied a mouse model of intestinal allergy (22). In contrast to nonvaccinated mice, MVA-OVA vaccination significantly prevented allergic symptoms and inflammation, while wtMVA vaccination did not ameliorate intestinal allergy. Beneficial effects were associated with a significant reduction in OVA-specific IgE levels, which was achieved upon challenge of mice in the acute allergic phase. This suppressive effect was observed even in MVA-vaccinated mice, but was more prominent after MVA-OVA vaccination. Furthermore, suppression of IgE in MVA-OVA vaccinated mice was accompanied by a significant induction of OVA-specific IgG2a production, preferentially detected after food challenge. Finally, the increased IgG2a/IgG1 and IgG2a/IgE ratios indicated a shift toward a Th1-biased immune reaction. These findings were further supported by strongly reduced IL-4 secretion from MLN cells, whereas IFN-γ levels were increased. MVA likewise induced IFN-γ secretion independent of a specific antigen. So far, the source of IFN-γ production was not determined. Thus, MVA-OVA vaccination impedes the development of allergic
symptoms, probably mediated by the induction of a systemic IgG2a antibody response and the induction of a local T_{H1}-biased cytokine response. Further studies need to be performed showing a direct evidence for IFN-γ producing CD8^+ T cells contributing to the beneficial effect. In addition, long-term efficacy of the preventive effect and applicability of MVA-based allergen vaccines for therapeutic interventions need to be investigated.

In summary, vaccination with MVA-OVA was proven to be a suitable strategy to prevent the onset of intestinal allergy in mice. The protective effect is likely mediated by induction of IFN-γ from CD8^+ T cells, which promotes the T_{H1}-biased immune response, for example, by induction of allergen-specific IgG2a. As MVA is a well-characterized vector system with a high safety profile that confers enhanced immunogenicity MVA-based vaccines can be suggested as suitable for the intervention of allergies.

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Author contributions

CB designed and carried out most of the experiments, analyzed data, and prepared the draft of the manuscript. AW performed and analyzed co-culture assays and supported in vivo experiments. S Schülke contributed to the study design and supported in vitro and in vivo experiments. MB provided the mouse model of intestinal allergy. YS and AS were involved in the preparation and characterization of MVA. HNA and SH performed histological analysis. ZW, MT, and SS substantially contributed to the conception of the study, the experimental design, interpretation of data, and supervised the study. GS, GR, and SV delineated the hypothesis, conceived, and initiated the study. All authors critically revised and approved the manuscript.

Conflicts of interest

S. Vieths declares fees received from the Food Allergy Resource and Research Program, Lincoln, NE, USA, the Institute for Product Quality, Berlin, Germany and Fresenius Academy, Dortmund, Germany (consultancy); the Medical University of Vienna, Austria (expert testimony); the American Academy of Asthma, Allergy and Immunology, the Deutsche Dermatologische Gesellschaft, the Spanish Society of Allergy and Clinical Immunology, Westdeutsche Arbeitsgemeinschaft für pädiatrische Pneumologie und Allergologie e.V., Köln, Germany, Gesellschaft für pädiatrische Allergologie und Umweltmedizin, and Ärzteverband Deutscher Allergologen (lectures); and Schattauer Allergologie Handbuch and Elsevier Nahrungsmittelallergien und Intoleranzen (royalties). His institution has received grants from Monsanto Company and Pioneer Hi-Bred International, Inc. for allergy testing of genetically modified plants; and travel/accommodation/meeting expenses from the German Research Foundation, the Federal Institute for Risk Assessment, the Austrian Society for Allergology and Immunology, the French Society of Allergology, the European Directorate for the Quality of Medicines and Health Care, the European Academy of Allergy and Clinical Immunology, the World Allergy Organization, the Technical University of Munich, the Deutscher Allergie- und Asthma Bund, the Association Monégasque pour le Perfectionnement des Connaissances des Médecins, the Federal Office of Consumer Protection and Food Safety, the German Chemical Society (GDCh), the Austrian Society for Dermatology and Venereology, and AKM Allergiekongress. The other authors of the paper declare no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

- **Figure S1.** Characterization of virus preparations.
- **Figure S2.** Vaccination regime.
- **Figure S3.** Vaccination with MVA-OVA prevents IgE secretion and induces IgG2a secretion in a mouse model of intestinal allergy.
- **Figure S4.** Vaccination with MVA-OVA prevents the reduction of food uptake in a mouse model of intestinal allergy.
- **Results S1.** Characterization of MVA and MVA-OVA preparations.
- **Results S2.** OVA-specific antibody response during the vaccination regime.
- **Data S1.** Material and methods.

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