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EXPERIMENTAL ALLERGY AND IMMUNOLOGY

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

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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 T_H2 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 T_H1- 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; INF, 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 basophil 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 T_H1 -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 T_H2 - to a T_H1 - 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 DyeFluor780, 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^{low}CD11b⁺CD11c⁺B220[−] myeloid dendritic cells (viable mDCs).

Co-culture of MVA-OVA-infected BMDCs and OVA-specific T cells

Bone marrow-derived dendritic cell (BMDCs) (5×10^5) were incubated for 2 h with wtMVA or MVA-OVA at a MOI of 1, washed, and co-cultured with 8×10^5 MACS-sorted splenic OVA-specific $CD8^+$ OT-I or $CD4^+$ OT-II T cells. As controls, cells were cultured with LPS (10 μ g/ml) or OVA (100 μ g/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 (10^8 pfu), MVA-OVA (10^8 pfu) or PBS, respectively. Three weeks after the final vaccination, mice were sensitized (i.p.) with 50 μ g 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 μ l/well, 100 μ g/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 Fc ϵ RI, 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 (4×10^5 /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 μ l/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

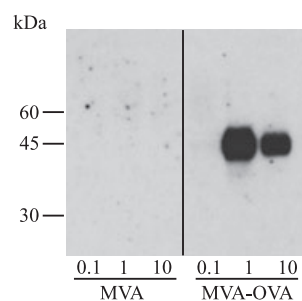


Figure 1 Infection with MVA-OVA induces OVA production in BMDCs. BMDCs were infected for 2 h with different MOIs of wtMVA or MVA-OVA, washed, and cultivated for 22 h. OVA expression in BMDC was analyzed by immunoblotting.

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 T $_H$ 1-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 T $_H$ 1

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

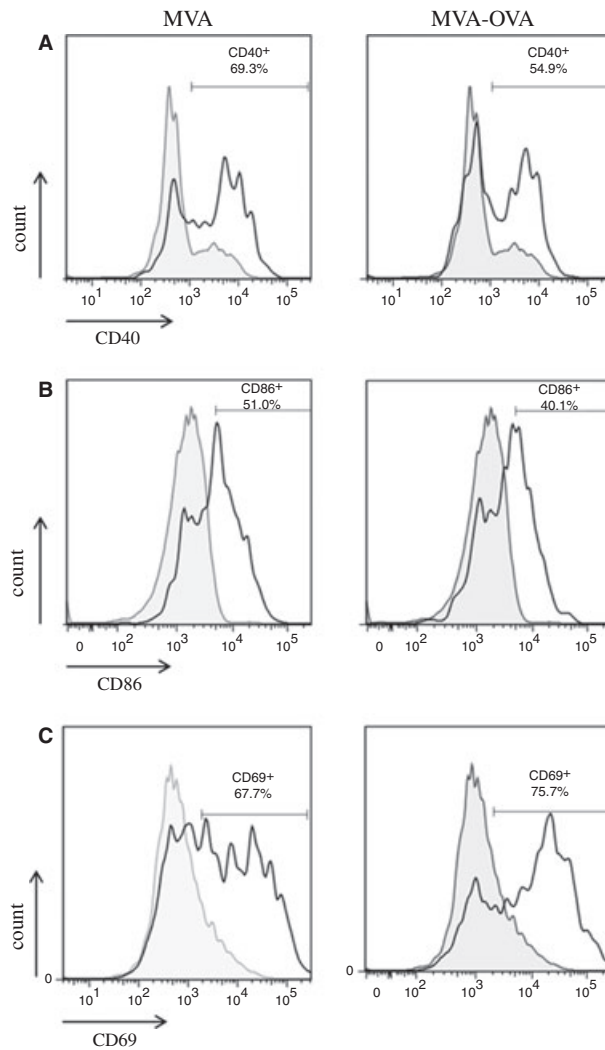


Figure 2 Wild type MVA (wtMVA) and MVA-OVA induces activation and maturation of BMDCs. BMDCs were infected for 2 h either with wtMVA or MVA-OVA at a MOI of 1. 24 h after infection CD40 (A), CD86 (B), and CD69 (C) expression on viable mDCs (viability dye^{low}CD11b⁺CD11c⁺B220⁺ cells) was analyzed by flow cytometry, respectively. Gray: not infected; black: infected.

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

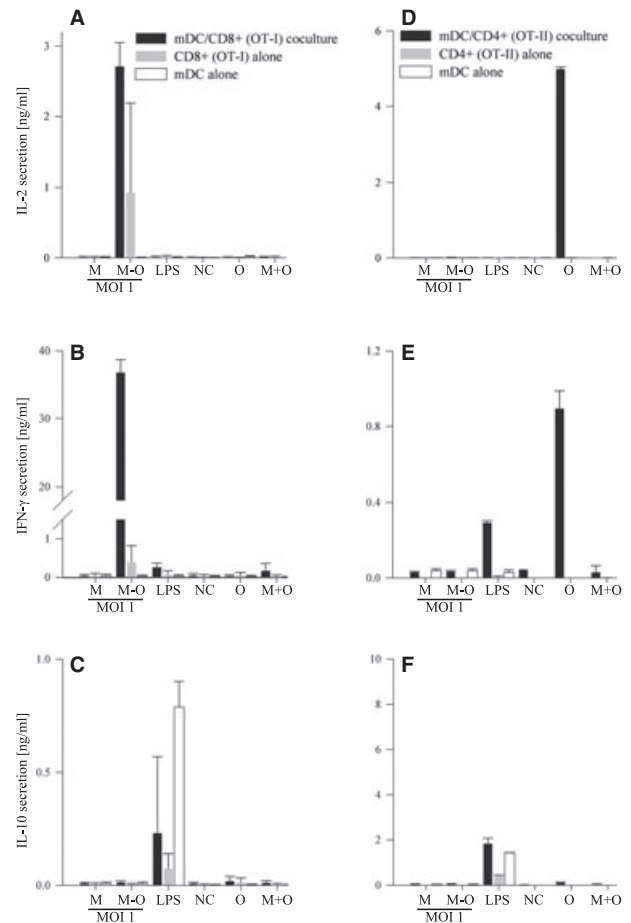


Figure 3 Modified vaccinia virus Ankara-ovalbumin (MVA-OVA) - infected BMDCs induce IL-2 and IFN- γ secretion from OVA-specific CD8⁺ T cells. BMDCs were incubated for 2 h with wtMVA (M, MOI 1) or MVA-OVA (M-O, MOI 1) and co-cultured with splenic OT-I CD8⁺ T cells (A–C) or OT-II CD4⁺ T cells (D–F). Untreated BMDCs (NC) and cells stimulated with LPS (10 μ g), OVA (O, 2.2 M) or wtMVA + OVA (M+O) were applied as controls. Secretion of IL-2 (A, D), IFN- γ (B, E), and IL-10 (C, F) into the supernatant was analyzed 24 h (IL-2) and 72 h (IFN- γ and IL-10) postinfection. Errors in A–C are standard deviation of 3 independent experiments, one representative experiment is shown for CD4 T cell cytokine secretion.

induce a systemic and local T_H1 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

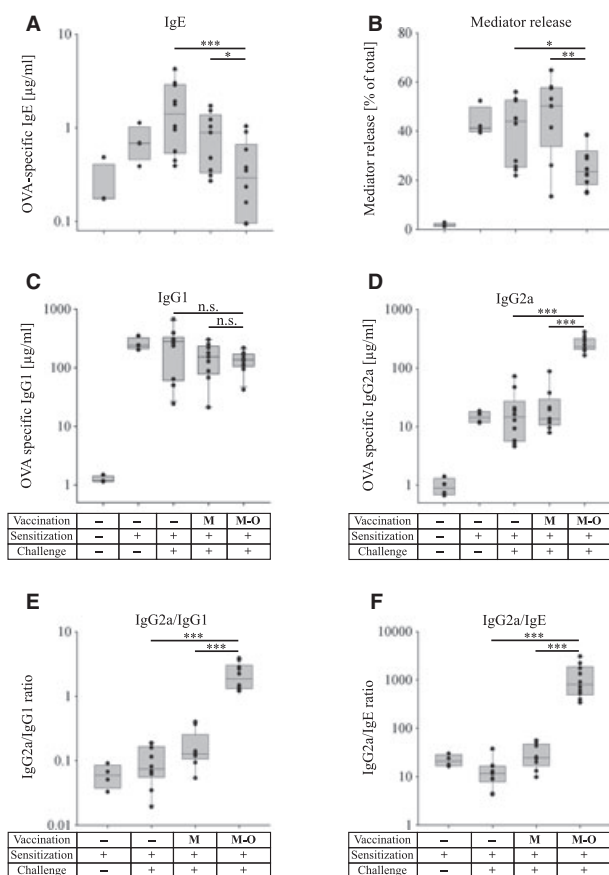


Figure 4 Vaccination with MVA-OVA induces a T_H1 immune response in a model of OVA-induced intestinal allergy. OVA-specific IgE antibody levels in BALB/c mice at day 6 of egg white challenge were determined by ELISA (A) and basophil activation assay (B). IgG1 (C) and IgG2a (D) levels were determined by ELISA and IgG2a/IgG1 (E), and IgG2a/IgE ratios (F) were calculated for individual mice. M: MVA, M-O: MVA-OVA.

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

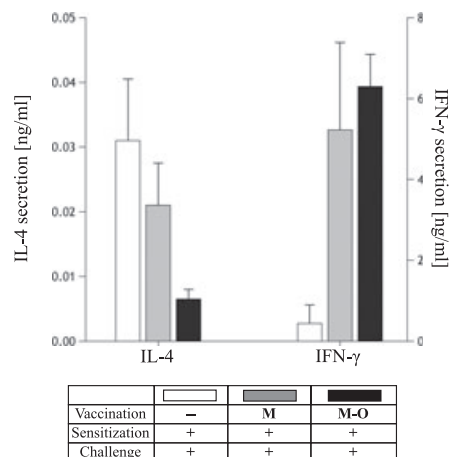


Figure 5 Vaccination with MVA-OVA induces a T_H1 immune response in MLN. MLN cells from allergic control mice, MVA, and MVA-OVA-treated mice were analyzed at day 6 of egg white challenge for IL-4 and IFN-γ secretion. Error bars indicate SD of 3 independent measurements.

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 T_H1 -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

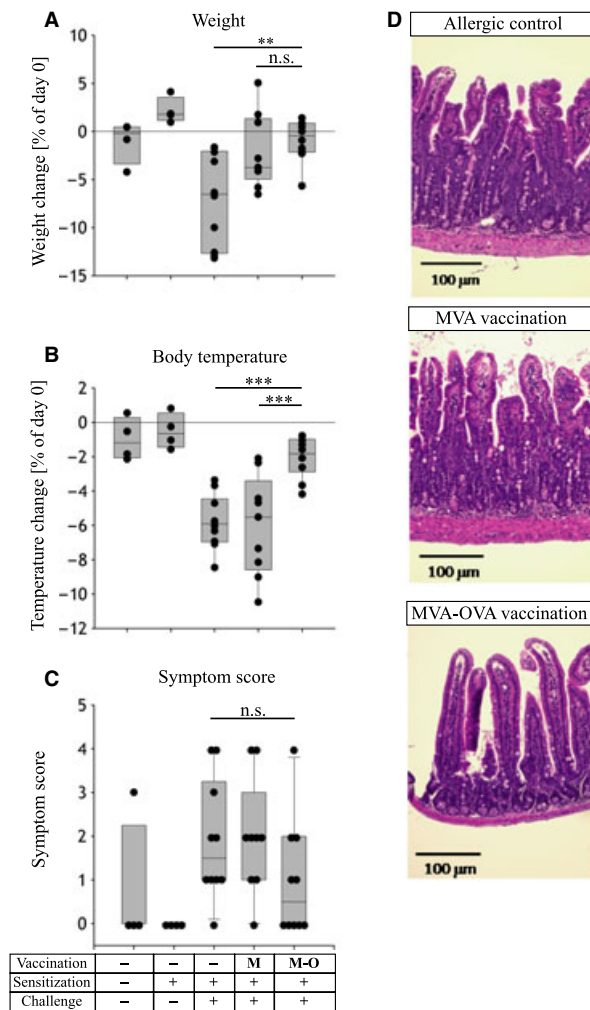


Figure 6 Vaccination with MVA-OVA prevents the onset of clinical symptoms and inflammation in a mouse model of intestinal allergy. (A) Body weight and (B) core body temperature at day 6 of egg white pellet challenge relative to day 0 were monitored. (C) Combined symptom scores (consistency of feces scored 0–3, roughness of fur scored 0–2) were taken in a blinded manner on day 6 of challenge. (D) Small intestines were harvested from allergic control mice, and MVA- or MVA-OVA-treated mice on day 6 of egg white pellet challenge for histological analysis. The data represent morphological changes of intestines in each group ($n = 5$).

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 T_H1 response. We showed MVA-OVA mediated IL-2 secretion as well as the induction of T_H1 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 Nörder 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 immune 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 INF- γ dependent manner.

To investigate the disease and immune-modulating properties of MVA-OVA, we applied a mouse model of IgE-mediated 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 T_H1 -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.

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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 Asthmabund, 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 Venerology, 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.

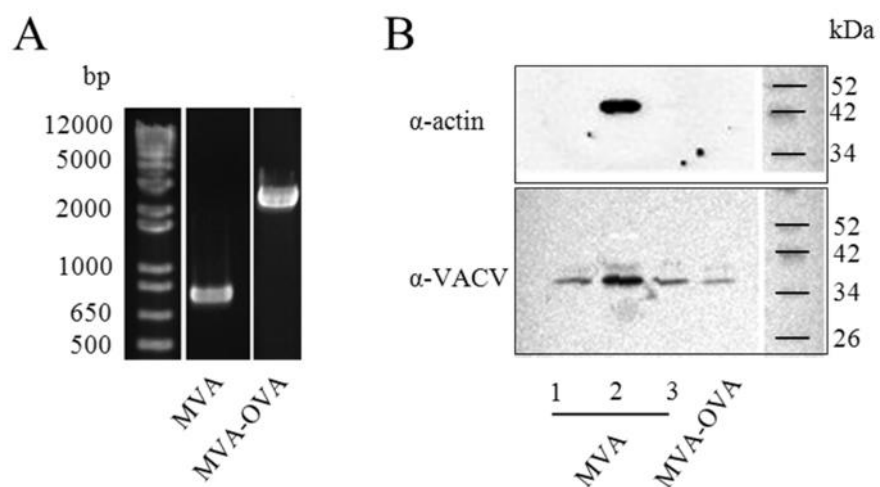
Results S1. Characterization of MVA and MVA-OVA preparations.

Results S2. OVA-specific antibody response during the vaccination regime.

Data S1. Material and methods.

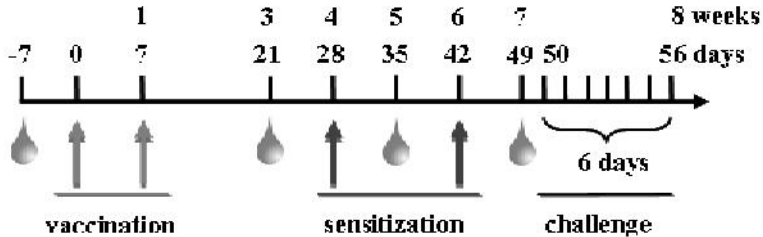
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Figure S1

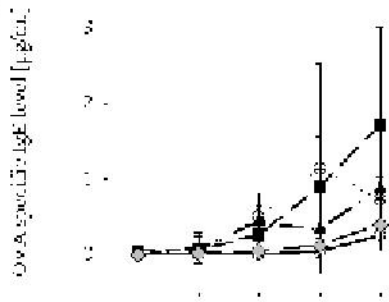




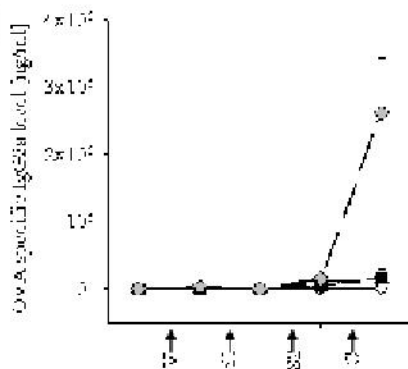
BALB/c



A



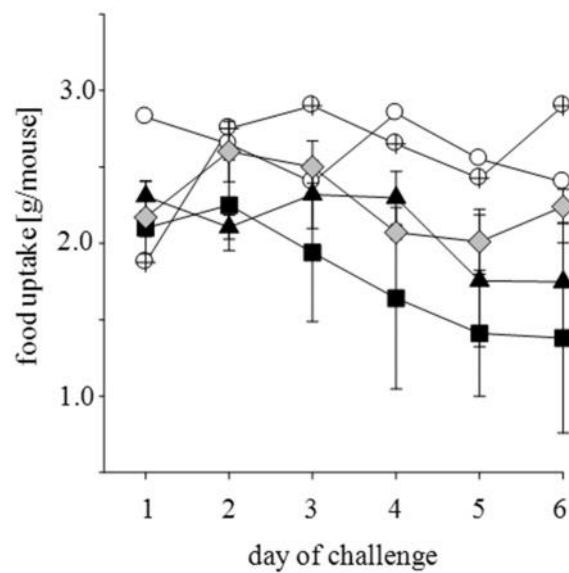
B



	○	⊕	■	▲	◇
sensitization		+	+	+	+
challenge	-	-	+	+	-

1
2
3

Figure S4



	○	⊗	■	▲	◇
vaccination	-	-	-	M	M-O
sensitization	-	+	+	+	+
challenge	-	-	+	+	+

4
5

Results

Characterization of MVA and MVA encoding OVA (MVA-OVA) preparations

Quality of virus preparations was tested by PCR and immunoblotting. PCR was performed on virus preparations using vector-specific primers encompassing the insertion site of the OVA gene. Both wtMVA and MVA-OVA virus stocks proved to be pure preparations (**Fig. S1A**). Purity was further assessed by staining MVA-OVA infected CEF cells with antibodies specific for MVA (raised against VACV) and OVA. The same number of plaques was stained with both antibodies, indicating that all infectious virions induce OVA expression (data not shown). Quality of virus preparation in terms of proteins derived from BHK cells (used for virus amplification) was analyzed by immunoblotting. Results indicate the absence of actin in wtMVA preparation no. 1 and 3, whereas a distinct amount of actin was detected in preparation no. 2 (**Fig. S1B**). Furthermore, among all batches, which have been normalized for same infectivity, preparation no. 2 showed the strongest reactivity with anti-VACV which reflects a high number of non-infectious virions. In order to avoid potential immune modulating residual cellular proteins, and to use highly infectious virions, wtMVA preparation no. 3 was selected for subsequent experiments. This preparation also had no detectable endotoxin content (< 0.05 EU/ml, data not shown).

OVA-specific antibody response during the vaccination regime

Induction of OVA-specific IgE antibodies by sensitization of non-vaccinated mice with OVA/alum was enhanced by subsequent challenge with egg white containing food pellets (**Fig. S3A**). Remarkably, MVA-OVA vaccinated mice showed low levels of OVA-specific IgE after sensitization similar to the non-sensitized control group. Mice vaccinated with wtMVA showed a reduced IgE response in comparison to non-vaccinated mice, but still higher IgE levels than MVA-OVA vaccinated mice. Interestingly, considerable amounts of OVA-specific IgG2a antibodies were only detectable after challenge with egg white containing pellets in MVA-OVA vaccinated mice (**Fig. S3B**).

Supporting information

Material and methods

Preparation and characterization of wtMVA and MVA encoding OVA (MVA-OVA)

Recombinant MVA encoding OVA was generated by El-Gogo *et al.* (21). Briefly, the gene encoding for OVA was inserted into the locus deletion III of wtMVA under the control of the vaccinia virus P7.5 early/late promoter. For amplification of virus and preparation of virus stocks, baby hamster kidney (BHK) cells were infected with an MOI of 0.03 for 2 days. Cells were lysed by 3 freeze-thawing cycles, the virus was harvested by 2 centrifugation steps and subsequently purified by sucrose gradient centrifugation. The virus titer was determined by plaque assay on chicken embryo fibroblasts (CEF) as described (33).

Purity of wtMVA and MVA-OVA preparations was analyzed by PCR using a primer pair (5'-GTACCGGCATCTCTAGCAGT-3', 5'-TGACGAGCTTCCGAGTTCC-3') encompassing the insertion site, immunostaining of plaque assays and immunoblotting. Plaque assay was done in duplicates for virus titer determination as described by Staib *et al.* (34). For the determination of virus stock purity, plaque assays were performed as above, but plaques were stained with rabbit anti-Vaccinia Virus (VACV) polyclonal Ab (Acris Antibodies, Herford, Germany), or with mouse anti-OVA monoclonal IgG1 (clone OVA-14, Sigma-Aldrich). Plaques stained for OVA and VACV were counted and compared. For immunoblotting 10^6 pfu of different virus proteins (wtMVA batches 1-3, and MVA-OVA) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Virus preparations were stained with rabbit anti-VACV polyclonal Ab (Acris Antibodies) and goat anti-rabbit polyclonal Ab conjugated to alkaline phosphatase (AP). After AP reaction the blot was washed with PBS + 0.05% Tween 20 and stained with mouse anti-actin monoclonal Ab and goat anti-mouse-HRP polyclonal Ab. Luminescence was detected by Lumi-Imager F1 (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Endotoxin content was measured by Endosafe-PTS (Charles River, Wilmington, USA).

Mice

C57BL/6J and BALB/c mice (6-10 weeks, female) were purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA). OT-I and OT-II mice on a C57BL/6J background were bred and maintained at the animal facility in Paul-Ehrlich-Institut. Animal experiments were carried out under specific pathogen free (SPF2) conditions and were performed in compliance to German regulations for animal welfare (trial F107/79).

MVA-OVA infection of murine derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were generated by cultivating bone marrow cells from C57BL/6 mice in the presence of GM-CSF (100 ng/ml) for 8 days. BMDCs (5×10^5 /ml) were incubated for 2 hours in 24-well plates with different MOIs (MOI of 1 and 10) of MVA-OVA or wtMVA. Cells were washed and incubated for 24 h (37°C, 5% CO₂, 95% humidity). For detection of OVA expression infected cells were suspended in PBS supplemented with protease inhibitors (EDTA free Protease Inhibitor Cocktail Tablets, Roche Diagnostics Deutschland GmbH, Mannheim, Germany), washed, and lysed by repeated freeze-thawing. OVA expression was analyzed in cell lysate and supernatant by SDS-PAGE and immunoblotting using a mouse anti-OVA monoclonal IgG1 (clone OVA-14, Sigma-Aldrich Chemie, Munich, Germany, 1:5000) and goat anti-mouse IgG-HRP (Sigma-Aldrich, 1:20000), and subsequent detection by LumiGLO Reserve (KPL, Maryland, USA).

Determination of OVA-specific antibodies

OVA-specific IgE, IgG1, and IgG2a antibody levels were analyzed by ELISA using OVA-coated microtiter plates (50 µl/well, 100 µg/ml). Plates were incubated with sera and standards for 2 h (IgG) or over night (IgE), washed, and incubated with (sub)class specific antibodies for 1 h. OVA-specific IgE was detected using a biotin-conjugated rat anti-mouse-IgE (R35-118, BD Biosciences) followed by incubation with streptavidin-HRP (30 min). OVA-specific IgG1 was detected by a HRP-conjugated goat anti-mouse-IgG1, OVA-specific IgG2a was detected by a HRP-conjugated goat anti-mouse-IgG2a (Invitrogen, Darmstadt, Germany). Mouse anti-OVA monoclonal IgE (E-G5, Chondrex, Redmond, USA), IgG2a (Lifespan Biosciences, Seattle, USA), and IgG1 (Sigma-Aldrich) were used as standards. HRP activity was detected by BD OptEIA TMB

substrate reagent kit (BD Biosciences). IgE blocking activity of sera from MVA-OVA vaccinated mice was investigated by competitive ELISA. Sera from human egg allergic patients selected for IgE-reactivity to OVA, were adjusted to obtain an OD value of approximately 0.8 and subsequently were pooled. Microwell plates were coated (4°C, o/n) with OVA (Sigma Grade V, 1 µg/ml), washed and incubated with pooled human sera and serial dilutions of sera from naïve or MVA-OVA vaccinated mice at (4°C o/n). Mouse anti-human IgE (Southern Biotech, 1:5.000) was used as secondary antibody, detection was performed for 20 minutes with TMB reagent, and OD_{450-620nm} was measured.

Histological analysis

Three cm segments of the jejunum (10 cm distal to the duodenum) were harvested, fixed in 10% formalin and embedded in paraffin. Three or 5 µm thick sections were prepared and treated with hematoxylin & eosin for morphological analysis (22).

Statistical analysis

Evaluation was performed with an analysis of variance (ANOVA), p-values were adjusted for multiple comparisons according to the Dunnett method. For symptom score the Wilcoxon rank sum test was used, adjusted according to the Bonferroni method. For statistically significant results the following convention was used: * – p-value < 0.05, ** – p-value < 0.01 and *** – p-value < 0.001. The statistical analysis was performed with SAS®/STAT software, version 9.3, SAS System for Windows.

Supporting information

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

Data S1: Material and methods

Results S1: Characterization of MVA and MVA-OVA preparations

Results S2: OVA-specific antibody response during the vaccination regime

Fig. S1: Characterization of virus preparations

Fig. S2: Vaccination regime

Fig. S3: OVA-specific IgE and IgG2a antibody levels over the treatment regime

Fig. S4: Food uptake during challenge of mice

