

Induction, treatment and prevention of eczema vaccinatum in atopic dermatitis mouse models



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ABSTRACT

Eczema vaccinatum is a severe and occasionally lethal complication of smallpox vaccine, characterized by systemic viral dissemination, distant from the initial inoculation site of the vaccine. A major risk factor for eczema vaccinatum is a background of atopic dermatitis, a chronic, common allergic, relapsing disorder, manifested by dry and inflamed skin, itchy rash, Th2 biased immune response and hypersensitivity to various antigens. Unlike the severe manifestations of eczema vaccinatum in humans, current models present only mild symptoms that limits examination of potential therapeutics for eczema vaccinatum.

The atopic dermatitis and eczema vaccinatum models we present here, are the first to simulate the severity of the diseases in humans. Indeed, dermatitic mice display persistent severe dermatitis, characterized by dry and inflamed skin with barrier dysfunction, epidermal hyperplasia and significant elevation of serum IgE. By exposing atopic dermatitis mice to ectromelia virus, we generated eczema vaccinatum that mimic the human disease better than known eczema vaccinatum models. Similarly to humans, eczematous mice displayed enlarged and disseminated skin lesions, which correlated with elevated viral load. Cidofovir and antiviral antibodies conferred protection even when treatment started at a late eczematous stage. Moreover, we are the first to demonstrate that despite a severe background of atopic dermatitis, modified vaccinia Ankara virus (MVA) vaccination protects against lethal ectromelia virus exposure. We finally show that protection by MVA vaccination is dependent on CD4⁺ T cells and is associated with significant activation of CD8⁺ cytotoxic T cells and induction of humoral immunity.

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1. Introduction

Atopic dermatitis (AD) is a chronic relapsing skin disease of complex etiology characterized by erythematous and pruritic rash affecting approximately 20% of children in developed countries. Active AD persists in most cases up to age 5, yet certain percentage of AD patients suffer from life-long relapsing AD symptoms [1,2]. Scaling of the skin, hypersensitivity to foreign antigens and aberrant activity of the immune system, are only few of several

abnormalities leading to Th2 biased immune response, accompanied by systemic allergic manifestations in AD patients. The aberrant immune response and the defects in dermal integrity present a major risk for AD patients to develop disseminated infections such as eczema herpeticum or eczema vaccinatum (EV) following exposure to herpes or vaccinia viruses (VACV) [2].

Vaccination with live VACV was used to eradicate smallpox. Today, immunization is recommended only for laboratory workers and first responders. AD is a contra-indication for vaccination due to the risk of EV. Yet, occasional EV cases appear involving long period of hospitalization and treatment with repeated high doses of VIG and antivirals [2–4].

EV is the most common severe pathology associated with smallpox vaccination that occurs in roughly 10–40 cases per million of either VACV immunized humans, or following a contact with vaccinee [5–7]. A background of active or history of AD have been associated with most cases of EV. Severe cases of EV involve virus

Abbreviations: EV, eczema vaccinatum; AD, atopic dermatitis; DNFB, 1-Fluoro-2,4-dinitrobenzene; ECTV, ectromelia virus; CDV, cidofovir; p.i., post infection; VACV, vaccinia virus; TEWL, trans epidermal water loss; MVA, modified vaccinia Ankara virus; VIG, vaccinia virus immunoglobulin; α -VACV, rabbit anti-VACV hyper immune serum; s.c., subcutaneously; i.n., intranasal; i.m., intramuscular; H&E, hematoxylin–eosin; BRP, biological reference preparation; MTTD, mean time to death; PRNT, plaque reduction neutralization test; d, day; h.p.i., hour post infection.

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dissemination and occasionally multi-organ failure with mortality rates of 30% when untreated [2].

Current animal models of EV, based on mild AD, exhibit only local skin lesions with rarely disseminated rash and does not involve systemic spread, unlike the human disease [7,8]. These limitations highlight the need to develop additional models of severe EV with disseminated phenotype in order to evaluate treatment.

In this study we established a robust AD model in mice by modifying a protocol of repeated sensitization with the hapten 1-Fluoro-2,4-dinitrobenzene (DNFB) on the back skin of two mouse strains, Nc/Nga and SKH-1 [9–11]. Nc/Nga mice are known for their vulnerability to develop AD either spontaneously or following allergen treatment [12,13]. SKH-1 mice are widely used in dermatologic research as immunocompetent and hairless mice, which allow easy manipulation of the skin as well as easy visualization of the cutaneous response [14].

Here, we show that DNFB-sensitized mice develop persistent severe dermatitis, characterized by very dry and red skin, loss of epithelial integrity manifested by an increase in the trans epidermal water loss (TEWL), epidermal hyperplasia and significant elevation in serum IgE levels. We further induced EV in AD mice by infection with ectromelia virus (ECTV) that resulted in severe disseminated rash, high viral load in the skin and internal organs with mortality rates of about 60–100%. These robust EV models were used to assess the therapeutic efficacy of Modified Vaccinia Ankara virus (MVA) vaccination and the therapeutic value of VACV-immunoglobulin (VIG), cidofovir (CDV) and anti-VACV hyper immune serum (α -VACV). We show that the best therapeutic treatment is a combination of CDV and α -VACV, which abrogated the viral load in skin and in internal organs, completely diminished skin lesions and prevented death. Delayed treatment (starting 8 days post infection, d.p.i.) of already eczematous mice, required high dose CDV treatment. Finally we show that despite the allergic AD background, pre- or post-exposure immunization with the smallpox vaccine MVA, is safe and protects against lethal respiratory poxvirus infection.

2. Materials and methods

2.1. Mice

Six-week-old female NC/Nga (Japan SLC, Shizuoka) or SKH-1 (Charles River, Germany) mice were housed in filter-top individual caging systems (IVC). General procedures for animal care and housing were done in compliance with the regulations for animal experiments at the Israel Institute for Biological Research. The end-points were weight loss (25% of the initial weight) and/or inability to respond to the righting reflex. Animals that reached these predetermined end-points were humanely sacrificed by cervical dislocation or by CO₂. ECTV challenge and MVA vaccination were performed on anesthetized mice (Ketamine 75 mg/kg, Xylazine 7.5 mg/kg in PBS).

2.2. Cells and viruses

Vero (ATCC CCL-8) and BS-C-1 (ATCC CCL-26) cells were maintained as recommended by ATCC.

Ectromelia strain Moscow (ATCC VR-1374) was propagated and titrated as described previously [15]. MVA clonal isolate F6 [16] at the 584th CEF passage was used and titrated as described previously [17].

2.3. Allergen sensitization and virus challenge

AD-like skin lesions were induced by repeated application of 100 μ l of 0.15–0.45% DNFB (Sigma-Aldrich, Israel) in acetone/olive

oil (3:1) to the back skin of mice once a week for 9 weeks prior to ECTV challenge and once a week for two weeks after the challenge. Control groups were treated with vehicle only (vehicle-sensitized).

ECTV infection: anesthetized mice were administered subcutaneously (s.c.) with 100pfu (100 μ l) or intranasally (i.n.) with 5000pfu (20 μ l).

MVA vaccination regimen: DNFB-sensitized mice (7th treatment), were vaccinated intramuscularly (i.m.) with 1×10^8 (50 μ l) PFU prime and boosted 3 weeks later. Two weeks after boost the mice were challenged with ECTV. Additional groups were vaccinated once at the indicated day. Vehicle-sensitized mice received the same regimen of MVA vaccination.

2.4. Measurements of TEWL and serum IgE

After the 9th DNFB-sensitization, total serum IgE level was quantified by ELISA (BD Pharmingen) according to manufacturer protocol. Trans epidermal water loss (TEWL) was measured using an evaporimeter (Tewameter TM300, Courage-Khazaka, Cologne, Germany). Water loss through the skin was measured with an open chamber probe applied to the skin. Baseline TEWL was measured in vehicle-treated mice.

2.5. Histopathologic analysis

Dorsal skin specimens were collected following the 9th DNFB treatment, 7 or 12 days after ECTV challenge, fixed in 4% formalin and embedded in paraffin. Skin sections (5 μ m) were cut and stained with either hematoxylin-eosin (H&E) or stained for virus using rabbit anti VACV polyclonal serum followed by anti-rabbit Alexa fluor 555-conjugated Ab, according to manufacturer instructions (Thermo Fisher Scientific). Tissue sections were evaluated at magnifications of X100 or X400.

2.6. EV scoring

Severity of lesion-progression was scored between 0 to 4 as follows: No lesions (0). Local lesion, smaller than 0.5 cm in diameter (1). Local lesion larger than 0.5 cm with or without few distant lesions (2). Disseminated lesions (3) or (4) depending on rash severity.

2.7. Determination of viral load in mouse organs and blood

Seven days post viral challenge, mice were bled. Liver, spleen, lung and skin were collected and kept at -80°C until analyzed. Organs were weighted, homogenized and viral load was determined by titration on BS-C-1 cells as previously described [18].

2.8. VIG, BRP and rabbit anti vaccinia hyper immune serum (α -VACV)

VIG (Omr IgG-am 5% VIG 50 mg IgG/ml, Omrix biopharmaceuticals Ltd.). BRP, a pharmacopeia European reference standard (human VIG BRP (biological reference preparation)) batch 1 was used to standardize the antibody titers to IU/ml. BRP potency is 23 IU/ml.

Hyper immune rabbit anti WR serum (α -VACV) was prepared as previously described [19].

2.9. Plaque reduction neutralization test (PRNT), comet inhibition and ELISA

PRNT was previously described [20]. Comet inhibition assay was performed as described previously [21]. ELISA to determine anti-vaccinia titer in the VIG, serum and BRP was done as described previously. Briefly, ELISA plates were adsorbed with β PL-inactivated

VACV IHD-J (whole virus, sucrose purified), blocked, reacted with serially diluted VIG, BRP or mouse serum followed by Alkaline-phosphatase conjugated anti human IgG (γ -chain specific) or anti mouse IgG (whole molecule) (Sigma 3187 and A5153 respectively) [19].

2.10. CDV, VIG and serum treatments protocols

Mice were administrated i.p. twice a week, for a total of 5 treatments with: VIG - 480 mg IgG/kg; CDV - 10 mg/kg or 100 mg/kg (high dose); α -VACV 0.3 ml; and as control similar volume of PBS or naïve rabbit serum (control).

2.11. In-vivo T cell depletion

Rat anti-mouse CD4 (clone GK1.5) and CD8 (clone 2.43) antibodies were produced and purified in our lab. anti-CD4 or anti-CD8 Abs were given (200 μ g per injection i.p.) one day (–1d) before MVA vaccination (which is equivalent to 2 days (–2d) before ECTV challenge). The treatment was repeated every 2–3 days during the experiment. Verification of CD4 or CD8 depletion was tested by flow cytometry (data not shown).

2.12. In vitro lymphocyte phenotyping by flow cytometry

To produce target cells, splenocytes of SKH-1 mice (+/– DNFB) were isolated (using gentleMACS Dissociator) and labelled with cellTrace Violet stain (life technologies) and excluded by FACS analysis. The cells were in vitro infected overnight with 1 MOI MVA. To produce effector cells, SKH-1 mice (+/– DNFB) were vaccinated with MVA (10^8 pfu i.m) and seven days later splenocytes were isolated (one day following target cells isolation). Target and effector cells were co-incubated in 96 U shape plate for 5hr in 37 °C 5% CO₂ in an E:T ratio of 1:5 (100,00 effector cells with 500,000 target cells). Next, the cells were extra and intra cellular stained using cytofix/cytoperm kit (BD). The following anti-mouse antibodies were used for flow cytometry (all are from e.Bioscience): anti-CD3 (clone 145-2C11) APC-Cy7, anti-CD4 (clone RM4-5) Alexa Fluor 700, anti-CD8 (clone 56-6.7) PerCP-Cyanine5.5, anti-IFN γ (clone XMG1.2) PE Cy7, anti-Granzyme B (clone NGZB) PE, anti-CD107a (clone 1D48), anti-D107b (clone eBioABL-93) both are eFluor 660. Flow cytometry was performed using FACS Aria 3 (BD) and analyzed by FlowJo software.

2.13. Statistical analysis

All statistical analyses were done by t-test using GraphPad Prism (GraphPad Software, San Diego, CA).

3. Results

3.1. Modeling atopic dermatitis in mice

AD was induced by repeated skin exposure to DNFB [9–11]. All DNFB-sensitized mice exhibited hallmarks of AD including dry and scaled skin (Fig. 1A,B & Supplementary Fig. 1A,B) with a significant increase in TEWL rate (Fig. 1C & Supplementary Fig. 1C) and serum IgE (Fig. 1D & Supplementary Fig. 1D). Notably, the increase in TEWL and IgE levels upon DNFB treatments was much higher in SKH-1 than Nc/Nga mice.

A 10-fold increase in the epidermal thickness and extensive crusting following DNFB sensitization was accompanied by dermal increased cellularity (dermatitis) (Fig. 1E–J).

3.2. Establishing eczema vaccinatum model in mice

Exposure of AD patients to VACV may result in EV, characterized by disseminated dermal viral lesions and virus spread to internal organs. Exposure of AD mice (both Nc/Nga and SKH-1 mice sensitized with DNFB) to VACV resulted in local lesion with occasionally limited disseminated rash (data not shown & [7,8]). We hypothesized that limited dermal dissemination of VACV in mice reflects differences in host susceptibility to VACV between mice and humans. We therefore infected our AD-mice with ECTV, a natural mouse pathogen closely related to VACV [22]. Progression in lesions, morbidity and survival of the mice were monitored and scored. Following ECTV infection, control mice showed very small and local dermal lesions (Fig. 2A,C). Most of the mice in this group did not exceed EV-score 2 and mortality rates were low (Fig. 2E,F). In contrast, infection of DNFB-sensitized mice provoked severe local lesions with massive dissemination (Fig. 2B,D Supplementary Fig. 2A,B) and increased mortality rates of 67% or 100%, SKH-1 or Nc/Nga, respectively (Fig. 2F or Supplementary Fig. 2C). It should be noted that progression of EV manifestations was faster in Nc/Nga compared to SKH-1 mice.

To confirm that distant dermal lesions represent viral dissemination and to correlate the role of viral dissemination to internal organs with disease severity, we measured the viral load in skin, spleen, lung, liver and blood of DNFB or vehicle-treated SKH-1 mice, 7 days post ECTV infection. Virus titers in all examined organs were significantly higher in DNFB-sensitized mice compared to vehicle-treated mice (Fig. 2G). In DNFB-sensitized mice, we observed hyperplastic acanthosis characterized by intercellular edema (spongiosis) with widening of intercellular spaces and intracytoplasmic viral inclusion bodies that were massively present in the epidermis (Fig. 2J,K). These manifestations were absent in vehicle treated animals (Fig. 2H,I). Next, to determine the distribution of ECTV in the skin we stained skin sections of ECTV infected, DNFB (Fig. 2N,O) or vehicle (Fig. 2L,M) treated mice with hematoxylin or with orthopoxvirus specific antibodies. Immunofluorescent staining revealed disseminated distribution of poxvirus infected cells both in the epidermis and dermis of DNFB-sensitized mice (Fig. 2N,O), that was barely detectable in vehicle treated mice (Fig. 2L,M).

To conclude, infection of DNFB-sensitized mice with ECTV resulted in robust and reliable phenotype of eczema, similar to human EV. Eczematous mice developed severe and disseminated skin lesions distant from the inoculation sites. SKH-1 mice showed reduced sensitivity to ECTV infection than Nc/Nga mice, as mortality rate in vehicle-treated mice was lower and MTTD in DNFB-treated mice was higher in SKH-1 comparing to Nc/Nga mice. Altogether, both mouse models proved valuable, yet, the SKH-1 model was favored due to the hairless phenotype, lower sensitivity to ECTV and availability.

3.3. CDV and antibodies treatments reduce the severity of EV

Having established our EV models, we tested the efficacy of VIG, α -VACV and CDV treatments in both mouse models either as single or combined treatment. Control group received PBS and/or naïve rabbit serum (Figs. 3, 4, supplementary Fig. 3 and table 1). In the SKH-1 model, treatments 24 h.p.i with either α -VACV, CDV, combination of CDV + α -VACV or CDV + VIG prevented death completely, while treatment with VIG alone did not significantly affect mice survival ($p = 0.0389$, Fig. 3A). We further took advantage of the hairless phenotype, to compare the effect of these antiviral drugs on the dermal manifestations of EV. Treatments with α -VACV, CDV + α -VACV or CDV + VIG efficiently alleviated the dermal manifestations while CDV as a sole treatment delayed the manifestations but did not prevent them. VIG as a sole treatment alleviated the manifestations at late stages of the disease in surviving mice

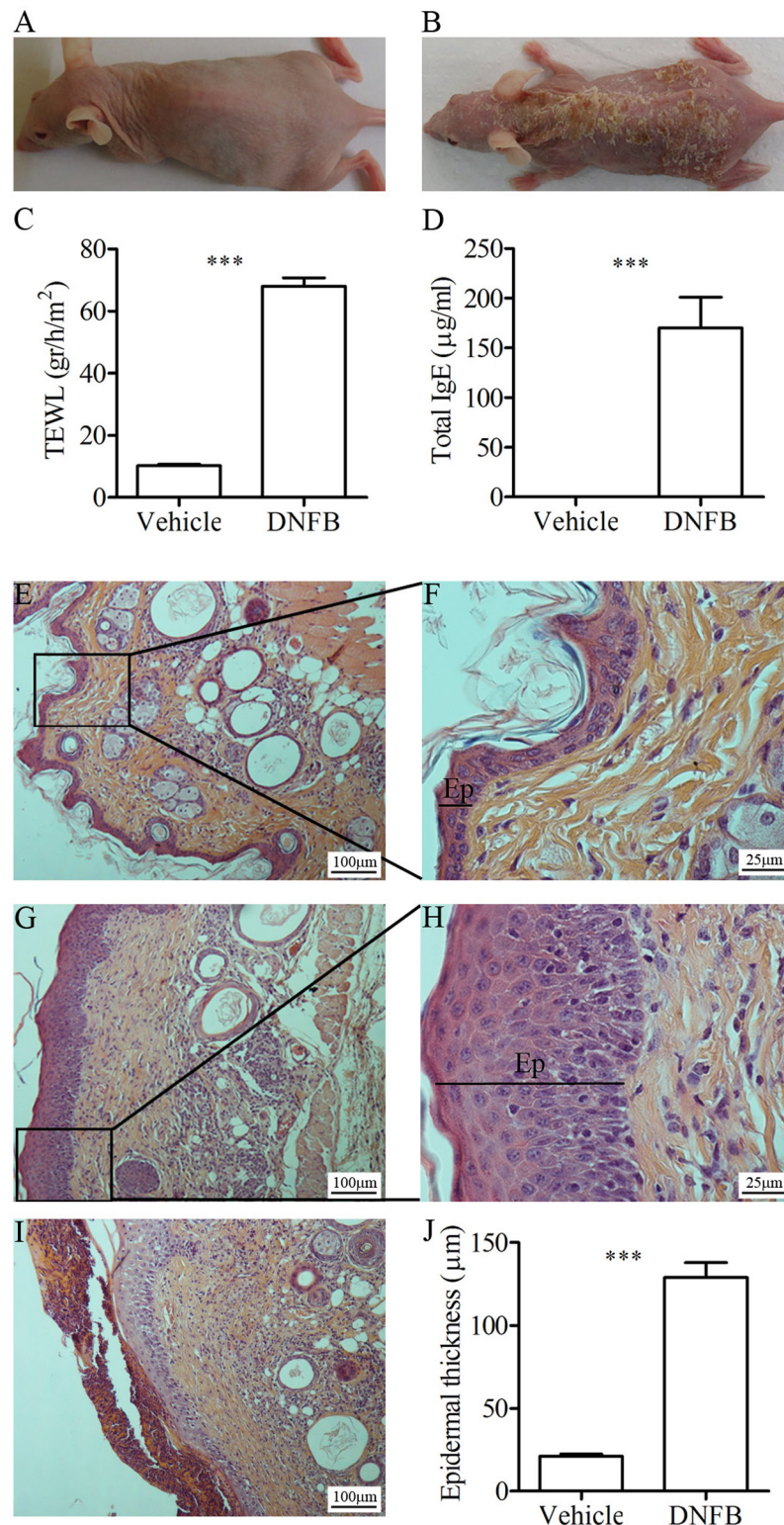


Fig. 1. Repeated application of DNFB induces AD symptoms in SKH-1 mice. Skin condition of (A) vehicle or (B) DNFB-sensitized in SKH-1 mice. (C) TEWL measurements $n = 17$ mice in each group. (D) Total serum IgE $n = 4$ mice in each group. H&E staining of skin sections of (E, F) vehicle or (G–I) DNFB sensitized mice. Ep, Epidermis. (J) Epidermal thickness was determined by measuring the epidermis thickness of 8 areas in 5 different slides. Bars represent mean $\pm$ SEM. P value *** < 0.0001 .

compared to untreated mice (Fig. 3B). This can be attributed to the higher specific activity of α -VACV with superior neutralization index of both intracellular mature virions and extracellular virions (Table S2).

To further elaborate on the mechanism by which CDV and α -VACV conferred protection, we measured the level of serum anti

vaccinia IgG (25 d.p.i) in mice treated with CDV or α -VACV or control untreated. We show that protective CDV treatment reduced the mouse serum anti VACV IgG titer compared to untreated mice that survived the challenge ($p = 0.0027$). Furthermore, α -VACV treatment that prevented the eczema manifestations, significantly eliminated antibody production ($p < 0.001$) (Fig. 3C). We further

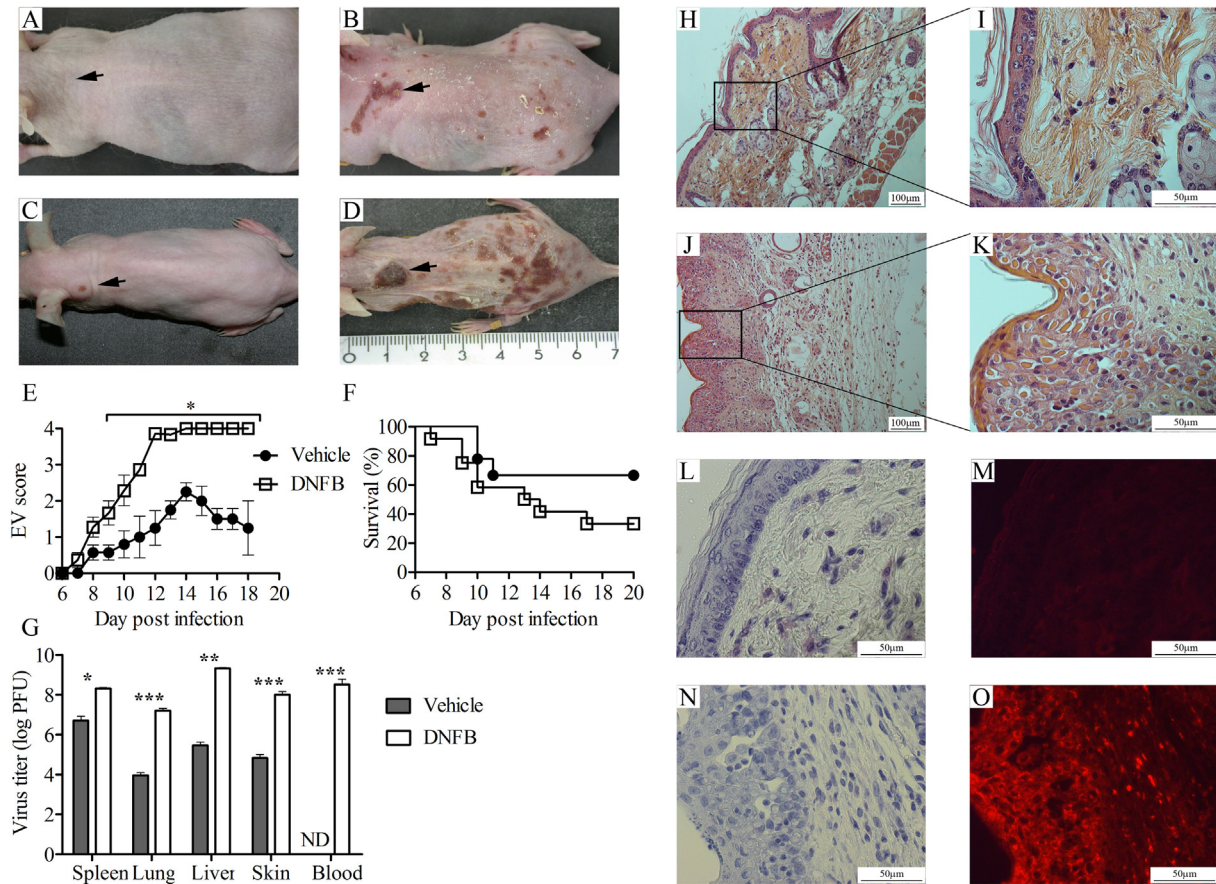


Fig. 2. Modeling EV by ECTV infection of DNFB-sensitized SKH-1 mice. ECTV infection of (A, C) vehicle or (B, D) DNFB-sensitized SKH-1 mice. Pictures were taken 8 (A, B) or 12 d.p.i. (C, D). Arrows indicate infection spots. Time course of skin clinical EV score (E) and survival rate (F) of ECTV infected mice (vehicle $n = 9$ mice, DNFB $n = 12$ mice). (G) Viral load as log PFU/gr (or log PFU/ml, for blood) was measured 10 d.p.i. ND, not detectable $n = 4$ mice. (H–K) H&E back skin sections of ECTV infected, vehicle-sensitized (H, I) and DNFB-sensitized (J, K) mice. (L–O) Hematoxylin and anti VACV staining of skin sections from vehicle (L, M) or DNFB-sensitized (N, O) mice, 12 days post ECTV infection. Values and error bars represent mean $\pm$ SEM. P values: * < 0.05 , ** = 0.0094, *** < 0.0001 .

show that the IgE level was not affected by either CDV or α -VACV treatments suggesting that the effective treatments (CDV or α -VACV) directly affected viral replication and spread and not by indirectly alleviating the dermatitis background.

Having demonstrated that co-administration of CDV + α -VACV prevented death and eliminated the dermal manifestations of EV, we evaluated the effect of this treatment on the viral load in SKH-1 mice at different target organs 7 d.p.i.. At this time point, local lesions at the site of infection appear in virus-exposed, unprotected mice but not in protected mice (Fig. 3B). Indeed, a significant ($p < 0.0001$) reduction of 7–8 orders of magnitude was observed in the viral load in the spleen, lung, liver, skin and blood following the combined CDV and antibodies treatment started 24 h.p.i. (Fig. 3E).

To conclude, CDV and α -VACV efficiently ameliorated the disease manifestations as a sole treatment, yet, the combination of CDV with VIG or α -VACV performed better with the combination of CDV + α -VACV being the most effective treatment, controlling dermal and internal dissemination and preventing mortality. Similar results were also observed in the Nc/Nga mouse model (Supplementary Table 1).

3.4. High dose CDV rescues EV symptomatic mice

To address the challenge of treating symptomatic EV mice, we evaluated the efficacy of CDV and α -VACV treatments starting 1, 3 or 5 d.p.i. by means of EV-score and survival. CDV, better than

α -VACV, prevented death (CDV started at days 1 or 3 prevented death completely, when treatment with CDV started 5 d.p.i., 75% survival was observed while, treatment with α -VACV rescued 100%, 75% or 50% of mice when started 1, 3 or 5 d.p.i., respectively, Fig. 4A,B). However, their combination was preferable (Fig. 4C) with 100% survival even if treatment started on day 5. Effective treatment, on days 8 or 10, when viral load was high (Fig. 3E) and mice were eczematous, was achieved by high human equivalent dose CDV (100 mg/kg) (Fig. 4D). For all treatments, delayed onset resulted in increased morbidity, yet, α -VACV alone or in combination, at early days alleviated morbidity better than CDV (compare Fig. 4B and C to A). VIG alone was a poor sole treatment regardless of timing (data not shown).

3.5. MVA vaccination elicits protective immune response in AD mice

MVA is a highly attenuated vaccine strain being evaluated as a safe smallpox vaccine [23]. In case of smallpox reemergence, individuals with severe AD will be at high risk of developing EV if vaccinated with VACV and of smallpox if unvaccinated. To evaluate whether under the severe AD immune bias, MVA vaccination maintains its safety and efficacy, even in a challenging setup of short-term or post-exposure prophylaxis, we vaccinated AD-mice with MVA 3 days prior, one day prior or one day post-ECTV infection. All MVA vaccinated mice survived ECTV infection (Fig. 5A). Furthermore, MVA vaccination 3 days before ECTV inoculation completely prevented skin manifestations and even vaccination

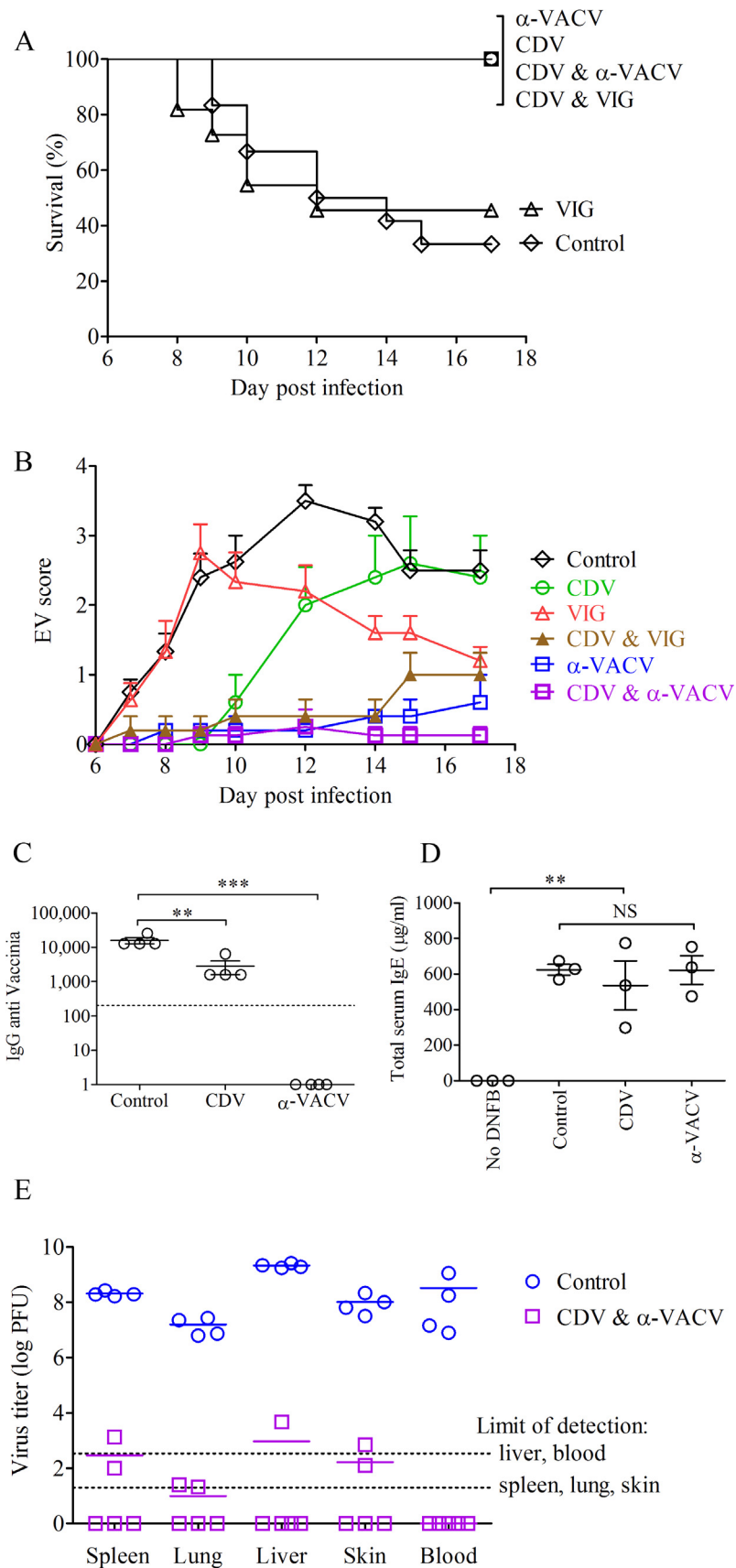


Fig. 3. Efficacy of CDV, α -VACV and VIG treatments in EV SKH-1 mice. DNFB-sensitized SKH-1 mice were infected with ECTV. 24 h.p.i. mice were administrated with the indicated treatments (CDV-10 mg/kg). (A) Survival curve, (B) Time course of skin clinical EV score. In control group $n = 12$ mice, VIG treated group $n = 11$ mice, CDV & α -VACV $n = 8$ mice, in CDV or α -VACV or CDV & VIG $n = 5$ mice. Measurements of levels of serum anti vaccinia IgG (C) and total serum IgE (D) 25 d.p.i. (E) Viral load seven d.p.i. of mice treated with combination of CDV and α -VACV or control treated mice. Lines represent mean and dotted lines represent limit of detection.

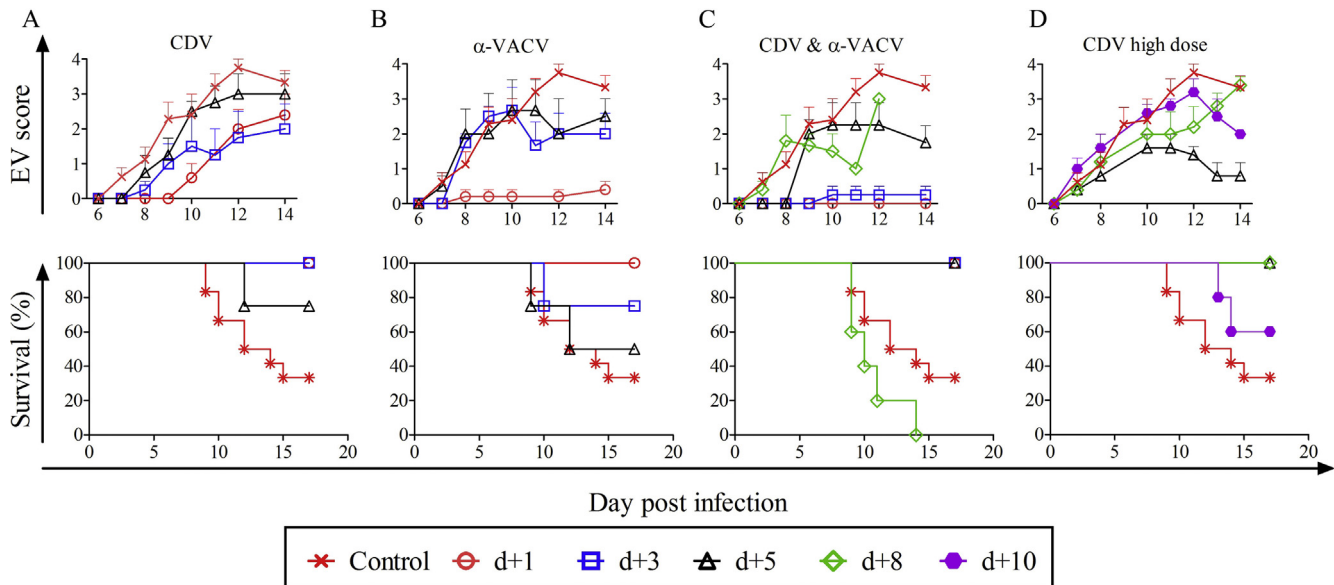


Fig. 4. Delayed treatment efficacies of CDV or antibodies in EV-SKH-1 mice. SKH-1 mice were sensitized with DNFB for 9 weeks, followed by ECTV infection. At different time points p.i. (1–10 days) mice were administrated with (A) 10 mg/kg CDV, (B) α -VACV, (C) combination of CDV and α -VACV or (D) 100 mg/kg CDV. In control group $n = 12$ mice, in all other group $n = 4$ –5 mice.

as late as 1 d.p.i. significantly alleviated skin manifestations (Fig. 5B).

To test whether CD4⁺ cells play a role in protection by MVA vaccination in this model, *in vivo* depletion of CD4⁺ cells was applied. anti-CD4 antibody efficiently depleted CD4⁺ cells (data not shown) and as can be seen in Fig. 5A and B, CD4⁺ depleted mice were no longer protected by MVA from challenge, substantiating their crucial role in protection by MVA vaccination. Using the same procedure, we failed to deplete CD8⁺ cells using the same antibodies that efficiently deplete CD8⁺ cells in C57BL/6 mice (data not shown).

Analysis of splenic CD8⁺ T cells revealed that MVA vaccination induced expansion (number) and activation (number of CD8⁺IFN γ ⁺ cells as well as number of CD8⁺GrzB⁺ and CD8⁺CD107a/b⁺) in non-AD mice. In AD mice, MVA vaccination does not induce splenic CD8⁺ expansion yet, it significantly induces activation of CD8⁺ cells (IFN γ ⁺ or GrzB⁺ or CD107a/b⁺) (Fig. 5C,F and data not shown).

Next we examined the induction of humoral response in MVA vaccinated AD mice compared to non-AD mice, and found that the level of anti-vaccinia IgG antibodies was comparable to vehicle treated mice following prime and boost vaccination (Fig. 5G).

Finally, we asked whether MVA vaccination induce sufficient protective immunity to withstand respiratory orthopox exposure despite the immune bias resulting from AD. To address this question AD SKH-1 mice were vaccinated with MVA either pre or post exposure to lethal respiratory ECTV challenge, representing smallpox [18]. We show that MVA vaccination pre or even at the day of challenge, protected from death (Fig. 5H). Indeed, IgM and IgG anti-VACV titer was 8–16 times higher (respectively) in AD mice vaccinated with MVA 2 days pre-challenge (data not shown) compared to unvaccinated ECTV challenged AD mice. Altogether, these results demonstrate that the protection afforded by MVA is mediated by both humoral and cellular response supporting the protective value of MVA as a smallpox vaccine for AD patients.

4. Discussion

EV is a severe complication of smallpox vaccines. A background of active or history of AD increases the risk of developing EV. Lack

of severe AD and EV animal models, limit the ability to evaluate therapeutics of EV. Availability of such models would allow selection of favorable treatments from already existing certified drugs and from future compounds, yet to be developed. For this reasons we sought after AD animal models that would exhibit AD symptoms resembling the human condition.

In this study we established two AD mouse models by repetitive sensitization with DNFB, a method previously described [10,24]. This sensitization provoked robust and severe AD with well-established hallmarks of AD. These manifestations include dry and scaled skin, epidermal thickening and significantly elevated TEWL and serum IgE level that altogether substantiate the relevance of this model as a model for human AD and set the basis to further develop an EV model.

Previous studies aiming to model EV in mice achieved very mild symptoms including minor enlargement of the “clinical take” and limited spreading of viral lesions. These symptoms could not represent severe EV in humans. In this study, following infection of DNFB-sensitized mice with ECTV, the animals exhibited robust dissemination of viral lesions accompanied by viral spread to internal organs. Infection of AD mice with VACV did not result in severe dissemination ([25] and our unpublished data) but infection with ECTV did. It appears that in order to achieve a good animal model two prerequisites are needed: an AD host presenting skin abnormalities with Th2 biased immune response and a virus that can break the host immune defense mechanisms to induce human-like EV symptoms. Other EV mouse models utilized both mild AD symptoms and VACV which altogether resulted in mild EV manifestations. Additionally, the use of hairless mice in the present model allows better visualization and quantification of EV progression.

The mechanisms allowing uncontrolled viral dissemination in AD background are not fully understood. The combination of Th2 biased immunity, skin inflammation and barrier dysfunction are considered as major contributors of disease severity. In addition, high levels of nucleotides and other anabolic substrates in injured or inflamed skin further support efficient replication of poxviruses [2]. In the present work we demonstrate that DNFB sensitized mice exhibit barrier dysfunction and elevated IgE levels, a hallmark of AD and Th2 biased immunity. Our preliminary data further

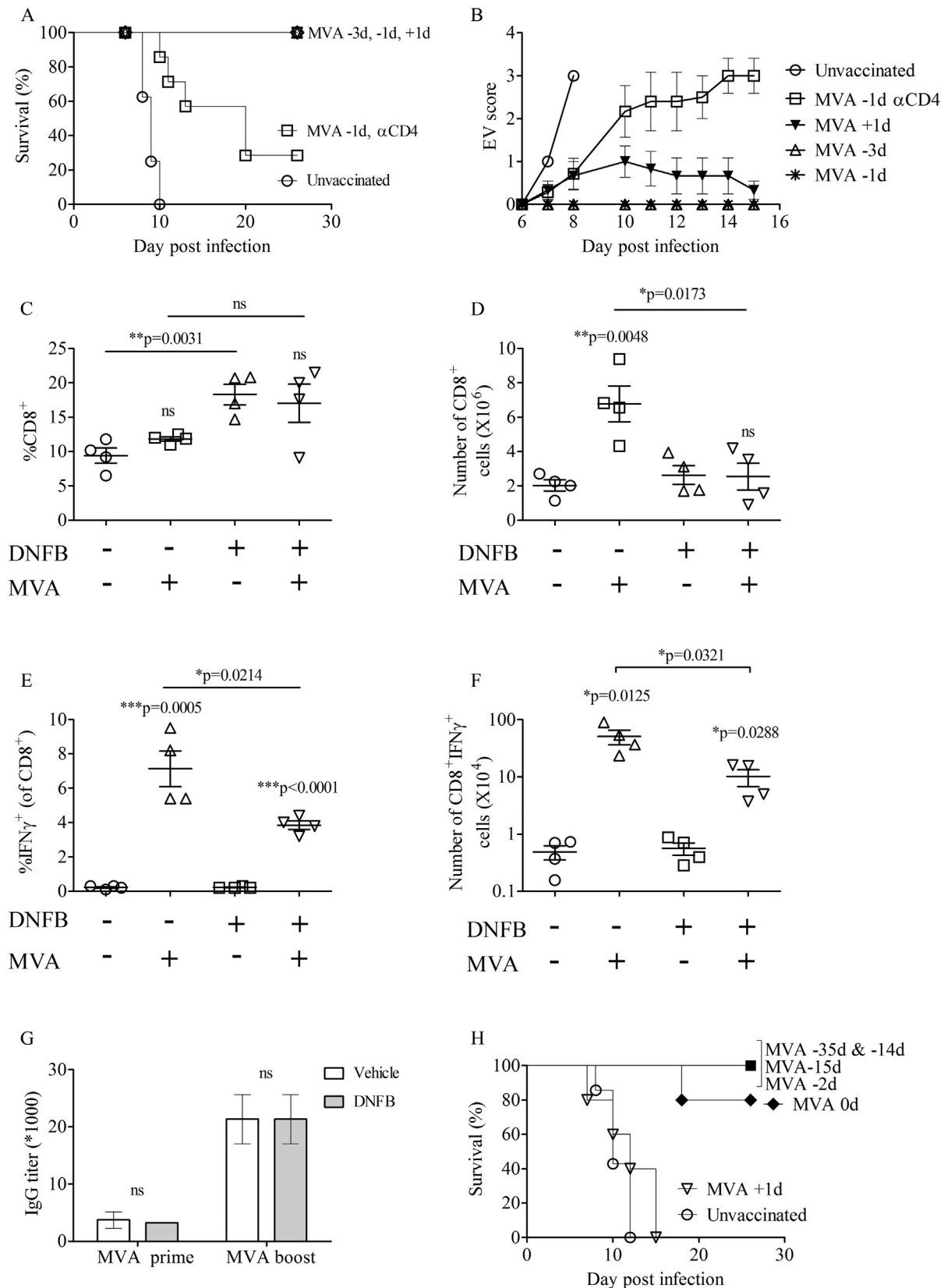


Fig. 5. Short-term MVA vaccination of AD mice protects SKH-1 mice in a CD4⁺ T cells dependent manner. Survival curve (A) and time course of skin clinical EV score (B) of ECTV infected DNFB-SKH-1 mice treated or not with a single dose of MVA which was administrated at the indicated day (d). Anti CD4 was administrated one day before the MVA vaccination and every 2–3 days during the experiment. n = 6–8 mice in each group. (C–F) Naïve SKH-1 mice (DNFB–, MVA–), MVA vaccinated (DNFB–, MVA +), DNFB-sensitized (DNFB +, MVA–) or MVA vaccinated following 9 weeks of DNFB-sensitization (DNFB +, MVA +) mice, were sacrificed and their spleens were homogenized for FACS analysis. (G) Measurement of IgG titer 20 days post prime (MVA prime) and 13 days post boost MVA vaccination (MVA boost) of vehicle or DNFB-sensitized mice. (H) Survival curve of ECTV i.n. infected AD mice which were vaccinated with MVA. The indicated day (d) represents the day of vaccination compared to infection day. n = 5–9 mice in each group. In C, D, E, F p values and n.s. refer to the comparison with the unvaccinated (MVA) group (adjacent left).

demonstrate induced expression of Th2 cytokines (e.g. IL-5, IL-10) in the skin of the AD mice. Indeed, robust virus replication in EV mice coincide with inflamed regenerating dermis (Fig. 2H–O), sustained elevation of Th2 cytokines (e.g. IL-5, IL-10, IL-13 and RANTES (CCL-5)). Indeed we demonstrated a high level of anti-vaccinia IgG in EV mice (Fig. 3C) which correlated with high virus titer and Th2 biased immunity. Several mechanisms may account for the induced humoral immunity in EV, mostly those involving Th2 effectors like IL-10. IL-10 induced in AD is a Th2 cytokine which enhances B cell survival, proliferation, and antibody production.

Currently, VIG is the first drug of choice for the treatment of EV [2]. However, the limited protective efficacy and availability of VIG highlight the need for additional therapeutic treatments. Evaluation of VIG treatment efficacy in a BALB/c mouse model of EV revealed that VIG administration one day post-inoculation with VACV, ameliorated the disease manifestations [26]. However, the severity of EV in this model was low when compared to the clinical manifestations in humans [4,27]. We therefore used our severe EV mouse model to evaluate the efficacy of various therapeutic treatments in prevention of EV. We show that VIG conferred partial protection when given 1 or 3 d.p.i. while α -VACV, exhibiting higher specific activity (PRNT, comet inhibition, Table S2) conferred full protection. As PRNT determines the neutralization efficacy of intracellular mature virions and comet inhibition represents the neutralization efficacy of extracellular-virus-spread, it is reasonable to suggest that similarly to protection against virulent poxviruses [19] also in EV, both neutralization activities contribute to protection against eczema. Interestingly, although both serum and CDV conferred full protection against EV-induced mortality α -VACV treatment ameliorated the dermal manifestations better than CDV when administration started at early stages of the disease (e.g. Fig. 4A, B day 1). It is reasonable to suggest that α -VACV treatment efficiently eliminated the virus and as a consequence virus specific IgG was not produced while CDV was less effective in virus elimination and indeed antibody production was less affected (Fig. 3C). None of the above treatments affected the AD state as IgE levels remained unchanged (Fig. 3D).

As expected, once the onset of treatment was postponed, the efficacy of sole treatment declined. Nevertheless, combination of CDV and α -VACV succeeded in rescuing 100% of animals even when treatment started as late as 5 d.p.i. indicating that at this stage of disease, protection depends both on inhibition of viral DNA replication by CDV, and on prevention of viral spread by antibodies. However, complete protection of severely eczematous mice (8 or even 10 d.p.i.) was achieved using human-equivalent high dose-CDV.

Treatment of EV was recently achieved by the combined use of multiple antivirals including: VIG, CDV (and its oral derivative CMX001) and Tecovirimat. Yet, their relative contribution is unknown [4]. We suggest that the models presented here would allow better evaluation of existing and new antivirals to treat EV.

Our data show the superior effect of α -VACV over VIG treatment to rescue and reduce the symptoms of severe EV in our mouse model. This observation can be attributed to the higher specific activity of α -VACV with superior neutralization index of both intracellular mature virions and extracellular virions (Table S2). These results suggest that development of a high-affinity α -VACV antibody-based product could improve the bank of medical treatments for human EV cases and therefore should be considered.

If smallpox reemerges, the vulnerability of AD patients to viral infections should be accounted. Exclusion of AD people from smallpox vaccination campaign while their close contacts are vaccinated with VACV will expose these individuals to a dual risk of either being infected with smallpox or developing EV from their close contacts that were vaccinated with VACV. Based on the severity

of EV in VACV exposed AD patients it is reasonable to suggest that smallpox in AD population would be severe and therefore the ability to prevent it by MVA vaccination is appealing. Clinical trials with MVA demonstrated the safety and immunogenicity of MVA in vaccination of patients with AD at various stages and severities, mainly mild and intermediate [28]. Our study further shows that MVA vaccination on a background of severe AD is not only immunogenic but also protective both against lethal respiratory exposure, representing smallpox but also as an effective post exposure prophylaxis of EV (e.g. in a case of undesired VACV exposure of AD patient). Protection against lethal poxvirus (VACV WR) intranasal infection with MVA vaccination 21 days pre exposure, has been previously demonstrated [25]. Yet, in the present work, we demonstrate the efficacy of short-term protection with MVA against lethal respiratory ECTV challenge, a mouse model of human smallpox. Interestingly, despite the strong Th2 biased immune background of the AD mice, our data clearly demonstrate that following MVA vaccination, humoral immunity was similarly induced in both AD and non-AD mice. Loss of protection following CD4 T cell depletion and activation of virus specific cytotoxic CD8 T cells further substantiated the ability of MVA to induce Th1 immune response despite the AD background. The SKH-1 mice has several advantages that allowed for the development of the AD and EV models and the evaluation of treatment modalities. Yet, analysis of mechanisms allowing induction of protective immunity in SKH-1 mice is complicated by the fact that SKH-1 are outbred and lack of genetic and immunologic tools. Even reagents like CD8 neutralizing antibody that perfectly works in C57BL/6 mice, does not neutralize CD8 cells in SKH-1 mice.

In summary, we established and characterized two robust AD and EV models, with which we demonstrated the efficacy of either antibodies, antivirals (CDV) or their combination. Finally, we demonstrate the ability of MVA vaccination to induce protective Th1 immune response in spite of the AD background.

Conflicts of interest

None.

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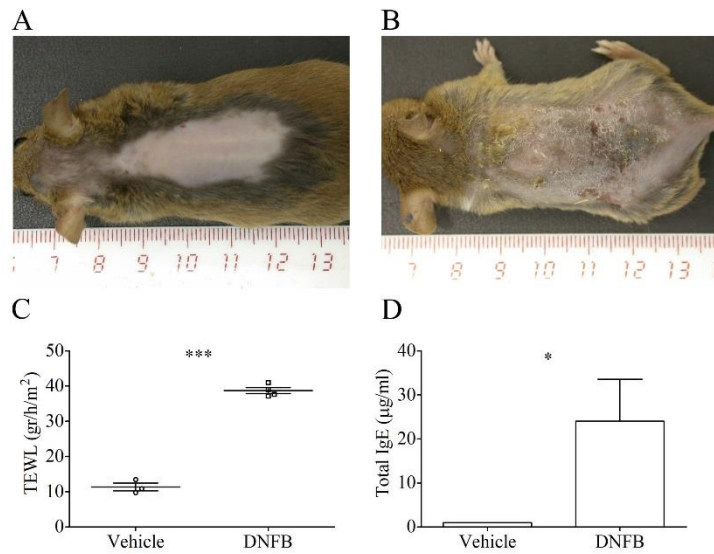
Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2017.06.014>.

References

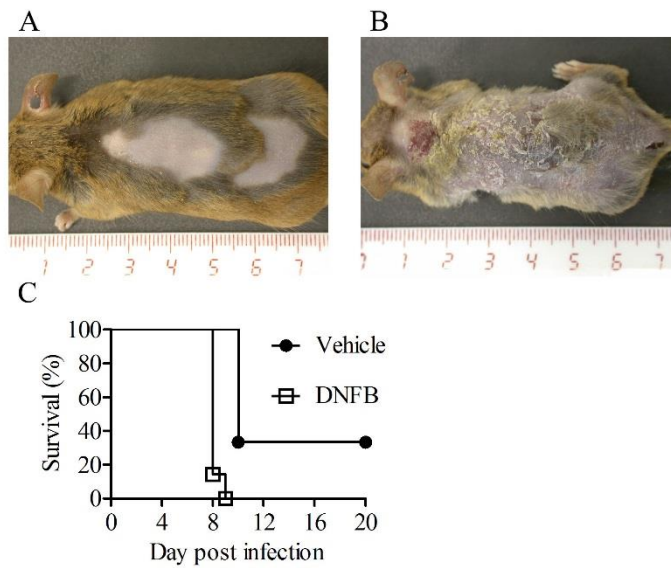
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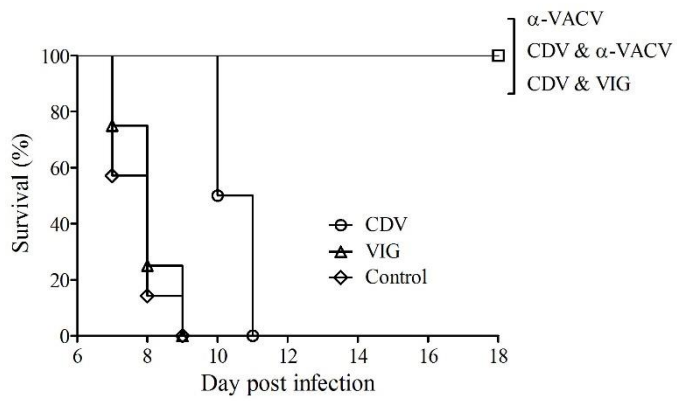
Supplementary Figure 1: **Repeated applications of DNFB induce AD model in Nc/Nga mice.**

Representative pictures of the vehicle (A) and the DNFB-sensitized (B) mice. TEWL (C) and total serum IgE (D) measurements. P values: *p<0.05, ***p <0.0001.



Supplementary Figure 2: **ECTV infection of DNFB-sensitized Nc/Nga mice induce EV symptoms.**

Nc/Nga mice vehicle (A) or DNFB-sensitized (B) were ECTV infected. Seven days post infection representative mice were pictured. (C) Survival curve of vehicle-treated or DNFB-sensitized mice infected with ECTV.



Supplementary Figure 3: **Combination of CDV with VIG, or α -VACV provide protection of EV Nc/Nga mice.**

DNFB-sensitized Nc/Nga mice were infected with ECTV. 24hrs p.i. the mice were administrated with indicated treatments.

Supplementary Table 1

	%Survival (MTTD)		
Treatment	day 1	day 3	day 5
Control	0 (8)	ND	ND
α -VACV	100	25 (25)	25 (14)
CDV	0 (11)	0 (10)	0 (9)
CDV & α -VACV	100	100	0 (17)
VIG	0 (8)	0 (7)	ND
CDV high dose	75 (18)	50 (11)	0 (8)

Supplementary Table 1: **Protection efficacy of different therapeutics in EV-Nc/Nga mice.**

AD-Nc/Nga mice were infected with ECTV and treated at different time points p.i. with the indicated treatments. Numbers in brackets represent MTTD.

Supplementary Table 2

	ELISA (end point titer)	Comet inhibition (NT50)	PRNT/ml (NT50)			PRNT IU/ml
	IHD-J	IHD-J	IHD-J	ECTV	Lister	Lister
VIG	51,200	320	124	785	1,699	89
α-VACV	1.64E06	1,600	1,451	4,705	11,789	616
BRP	8,063	<40	<20	<20	441	23

Supplementary Table 2: **Determination of anti-vaccinia titer in VIG and α -VACV.**

PRNT and ELISA were performed in order to determine the specific anti vaccinia antibodies in the VIG and α -VACV. The values for the ELISA are end point titer.