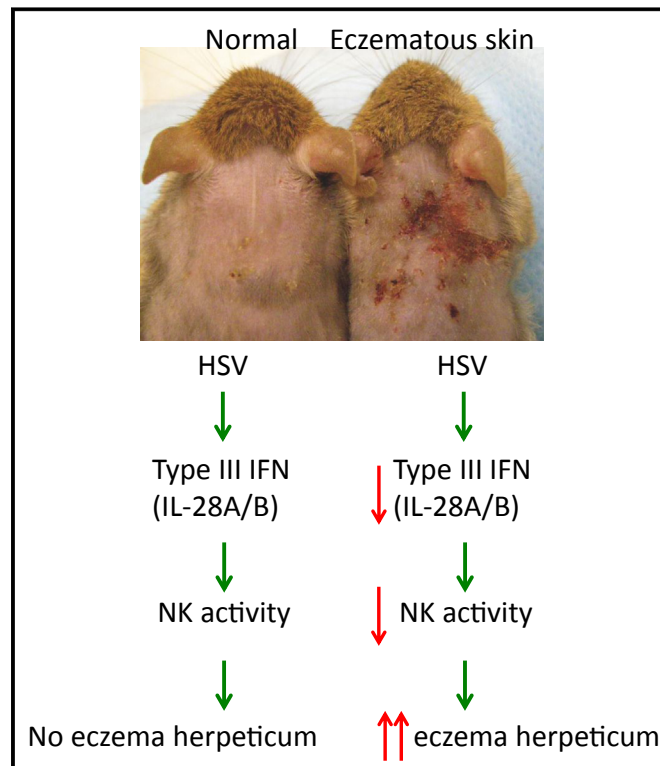


Defective natural killer cell activity in a mouse model of eczema herpeticum

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GRAPHICAL ABSTRACT



Background: Patients with atopic dermatitis (AD) are susceptible to several viruses, including herpes simplex virus (HSV). Some patients experience 1 or more episodes of a severe skin infection caused by HSV termed eczema herpeticum (EH). There are numerous mouse models of AD, but no established model exists for EH.

Objective: We sought to establish and characterize a mouse model of EH.

Methods: We infected AD-like skin lesions with HSV1 to induce severe skin lesions in a dermatitis-prone mouse strain of NC/Nga. Gene expression was investigated by using a microarray and quantitative PCR; antibody titers were measured by means of ELISA; and natural killer (NK) cell, cytotoxic T-cell, regulatory T-cell, and follicular helper T-cell populations were evaluated by using flow cytometry. The role of NK cells in HSV1-induced development of severe skin lesions was examined by means of depletion and adoptive transfer.

Results: Inoculation of HSV1 induced severe erosive skin lesions in eczematous mice, which had an impaired skin barrier, but milder lesions in small numbers of normal mice. Eczematous mice exhibited lower NK cell activity but similar cytotoxic T-cell activity and humoral immune responses compared with normal mice. The role of NK cells in controlling HSV1-induced skin lesions was demonstrated by experiments depleting or transferring NK cells.

Conclusion: A murine model of EH with an impaired skin barrier was established in this study. We demonstrated a critical role of defective NK activities in the development of HSV1-induced severe skin lesions in eczematous mice. (*J Allergy Clin Immunol* 2016;■■■:■■■-■■■.)

Key words: Atopic dermatitis, eczema herpeticum, herpes simplex virus, animal model, natural killer cell

Atopic dermatitis (AD; or eczema) is a chronically relapsing pruritic inflammatory skin disease. The cause of this disease is incompletely understood, but it is multifactorial, and the disease is manifested by complex interactions between genetic and environmental factors.^{1,2} Patients with AD are susceptible to infection with certain microbes, including viruses, such as vaccinia virus and herpes simplex virus (HSV). Thus they are

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Abbreviations used

AD:	Atopic dermatitis
EC-OVA model:	Mouse AD model induced by epicutaneous sensitization with ovalbumin
EH:	Eczema herpeticum
Foxp3:	Forkhead box protein 3
HSV:	Herpes simplex virus
NK:	Natural killer
qPCR:	Quantitative PCR
SEB:	Staphylococcal enterotoxin B
TEWL:	Transepidermal water loss
T _H :	Follicular helper T
Treg:	Regulatory T

excluded from smallpox vaccination because of their propensity to have eczema vaccinatum, a disseminated vaccinia infection.³ A subgroup of patients with AD can experience 1 or more episodes of a severe HSV infection of the skin. This condition is named AD complicated by eczema herpeticum (EH).⁴⁻⁶ EH is a rare but serious complication of AD. Clinical features of patients with AD who experience EH include severe disease, early age of onset, more frequent history of other atopic disorders, greater T_H2 polarity, allergen sensitization, and more frequent skin infections with other microbes.⁷ The primary predisposing factor for EH is HSV1 exposure, although HSV2 can cause EH in a minority of patients. Little is known about the cause of EH. However, a major defect that renders subjects susceptible to viral infection during AD might be an impaired epidermal barrier function.^{1,2} Alternatively, viral susceptibility can be caused by an imbalance between antiviral immune responses and regulatory T (Treg) cells⁸ and defects of the innate immune system.⁹

Several animal models of AD have been instrumental in our understanding of the pathogenic mechanisms of AD.^{10,11} For example, a mouse model induced by epicutaneous sensitization with ovalbumin (EC-OVA model) mimicked skin lesions of human AD characterized by epidermal and dermal thickening, infiltration of CD4⁺ T cells and eosinophils, and local expression of T_H2 cytokines.^{10,12} We also described another AD model in which repeated epicutaneous applications of a house dust mite (*Dermatophagoides farinae*) extract and staphylococcal enterotoxin B (SEB) induced eczematous skin lesions.¹³ This model has been used for testing potential therapeutics for AD treatment¹³ and for establishing and characterizing a murine model of eczema vaccinatum.^{14,15} In this study we sought to establish a murine model of EH and to characterize its clinical features and pathogenic mechanisms.

METHODS**HSV1 strains**

The KOS strain of HSV1¹⁶ and the green fluorescent protein-expressing KOS strain¹⁷ were provided by Dr Carl Ware, Sanford-Burnham Medical Research Institute. The McKrae strain (HSV1-McKrae)^{18,19} was provided by Dr Homayon Ghiasi, Cedars-Sinai Medical Center, and Dr Feng Yao, Brigham and Women's Hospital.

Mouse model of EH

NC/Nga mice²⁰ were used in all animal experiments. AD-like skin lesions were induced, as described previously.¹³ Briefly, mice were shaved on the back, and dermatitis was induced by 2 rounds of treatment with *D farinae*

extract (Greer Laboratories, Lenoir, NC) and SEB (Sigma-Aldrich, St Louis, Mo), followed by resting. AD scores of skin lesions were recorded based on the severity (0, no signs; 1, mild; 2, intermediate; and 3, severe) of 4 signs (redness, bleeding, eruption, and scaling), thus with a score of 12 in the most severe case. Seven days after the last *D farinae*/SEB administration, eczematous (with an AD score ≥ 8) and normal (sham-treated) mice were intradermally injected with 4.5×10^3 plaque-forming units (in 3 μ L) of HSV1 per site on the 4 sites at the center of skin lesions with skin pricking (15 times with a 27-gauge needle). A cohort (normal group) of age- and sex-matched mice with healthy skin was also infected at the same anatomic sites. EH scores are based on how many sites of HSV1 inoculation exhibit erosive skin lesions. Because 4 sites were inoculated, the highest EH score was 4. Scoring was performed by a blinded investigator. Animal experiments were approved by the Animal Use and Care Committee of the La Jolla Institute for Allergy and Immunology and conducted in the La Jolla Institute for Allergy and Immunology animal facility according to the guidelines in the "Principles of laboratory animal care" formulated by the National Society for Medical Research.

Measurement of transepidermal water loss

Transepidermal water loss (TEWL) was measured on shaved neck skin and back skin with a Tewameter TM 300 (CK Electronic GmbH, Cologne, Germany).

Histology

CD4⁺ and CD8⁺ T cells, Mac-1⁺ monocytes/macrophages, and Ly49G2 (4D11)⁺ and asialo GM1⁺ or NK1.1⁺ natural killer (NK) cells were detected based on results of immunochemical staining. Mast cells were stained with toluidine blue, and eosinophils and neutrophils were detected by using hematoxylin and eosin or Congo red staining.

Analysis of gene expression by using a microarray and quantitative PCR

Skin tissues were taken from infection sites or erosive areas on days 2 and 4 after infection by means of punch biopsy, and axillary lymph nodes and spleens were also isolated. Total RNA was extracted with TRIzol One Step RNA Reagent (BioPioneer, San Diego, Calif). The same amount of RNA from 3 to 5 mice was mixed for each cohort and cleaned by using the RNeasy Total RNA Mini Kit (Qiagen, Hilden, Germany). A microarray analysis was performed²¹ with 200 ng of total RNA from each cohort and SurePrint G3 Mouse Gene Expression 8x60K arrays (Agilent Technologies, Santa Clara, Calif), according to the manufacturer's instructions. Microarray data after infection will be deposited in the Gene Expression Omnibus on acceptance of this article (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSExxxxx>). These data were compared with data before infection (day 0) deposited previously in the Gene Expression Omnibus previously (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53132>).²¹

Total RNAs were also used as templates to prepare cDNAs. PCR reactions were performed with primer sets successfully used in previous publications (sequences are available on request). PCR products were analyzed by means of agarose gel electrophoresis. Quantitative PCR (qPCR) was performed with LightCycler 480 (Roche Applied Science, Mannheim, Germany). Viral titers were also measured by using qPCR analysis of HSV1 *pol* compared with 18S RNA.

NK cell experiments

Depletion of NK cells 1 day before HSV1 infection was performed with anti-asialo GM1 (Wako Pure Chemicals, Richmond, Va) or normal rabbit IgG (Cell Signaling Technology, Danvers, Mass) or anti-NK1.1 mAb (BD PharMingen, San Jose, Calif) or control rat IgG_{2a} (BD PharMingen), as described previously.¹⁴ Skin tissues were harvested on days 0 (before infection), 2, 4, and 7 after infection. Splenocytes from NC/Nga mice were negatively selected by using the EasySep Mouse NK cell Enrichment Kit

(STEMCELL Technologies, Vancouver, British Columbia, Canada). These NK-enriched cells were cultured in IL-15 (PeproTech, Rocky Hills, NJ) for 4 days. The cultured NK cells (purity $\geq 93\%$) were checked by using flow cytometry after staining with anti-NK1.1 and anti-CD3 (BD PharMingen) antibodies and intravenously transferred ($8\text{--}10 \times 10^6$ per mouse).

Flow cytometry

Single-cell suspensions of splenocytes or lymph nodes were surface stained with anti-NK1.1 and CD3. Then the cells were fixed, permeabilized, and stained with anti-granzyme B, anti-perforin, or anti-IFN- γ (BioLegend, San Diego, Calif). Data were acquired by using the BD LSRII (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, Ore). Follicular helper T (T_{FH}) cells and Treg cells in splenocytes or lymph nodes were identified as $CD4^+CD62L^-CD44^+CXCR5^+Bcl6^+$ and $CD4^+CD62L^-CD44^+Foxp3^+$ cells (antibodies were all from eBioscience, San Diego, Calif, unless otherwise noted), respectively. CXCR5 is stained by using 3 steps: cells were incubated with purified anti-CXCR5, followed by biotinylated goat anti-rat IgG (Jackson ImmunoResearch, West Grove, Pa), and then by streptavidin conjugated with phycoerythrin-Cy7 (eBioscience).

Treg suppression assay

$CD4^+$ cells were enriched from the spleens of normal mice by using $CD4$ Microbeads (Miltenyi, San Diego, Calif). $CD4^+$ cells were further sorted by using fluorescence-activated cell sorting for $CD4^+CD45RB^{\text{high}}$ cells (responder cells). Treg cells were sorted as $CD4^+CD25^{\text{high}}$ cells from the spleens of either eczematous or normal control mice. Antigen-presenting cells were isolated as $CD11c^+$ cells from the negative fraction of $CD4$ purification by using $CD11c$ Microbeads (Miltenyi) and further X-irradiated at 3000 rad before use. Responder cells (1×10^5) were prelabeled with CellTrace Violet (Life Technologies, Carlsbad, Calif) and mixed with either eczematous or normal Treg cells at different ratios and then stimulated with $5 \mu\text{g/mL}$ soluble anti- $CD3\epsilon$ antibody in the presence of 1×10^5 antigen-presenting cells isolated as above to analyze Treg cell-mediated suppression of responder cell proliferation and division. Based on the dilution of CellTrace Violet analyzed with a flow cytometer, proliferation index, division index, and percentage suppression of proliferation index and division index were calculated with FlowJo software.

ELISA

ELISA was performed on mouse sera with the HSV1 envelope glycoprotein gD (Abcam, Cambridge, United Kingdom) as a capturing agent. Mouse sera were incubated, and IgG bound to gD was detected by using horseradish peroxidase-conjugated anti-mouse IgG (BD PharMingen) antibody, followed by incubation with TMB (BD Biosciences).

Statistical analysis

Statistical analysis was performed by using unpaired 2-way ANOVA or the Student t test with Prism software (GraphPad Software, La Jolla, Calif). A P value of less than .05 was considered statistically significant.

RESULTS

Clinical features of a murine EH model

We sought to develop a model of EH in dermatitis-prone NC/Nga mice.²⁰ First, eczema was induced by means of epicutaneous applications of *D. farinae* extract and SEB. Seven days after the last *D. farinae*/SEB administration, eczematous and normal (sham-treated) mice were infected with the McKrae strain of HSV1 (HSV1-McKrae)^{18,19} on 4 sites of shaved back skin through a combination of scarification and pricking. Skin lesions with bleeding and exudates started to develop on day 2 after infection at inoculation sites of eczematous mice. The lesions in eczematous mice peaked on days 8 to 10 and subsided

thereafter (Fig 1, A-C). By contrast, milder skin lesions occurred in only a few HSV1-infected normal mice. Analysis of hematoxylin and eosin-stained skin lesions before HSV1 inoculation showed a thickened epidermis and dermis with marked infiltration of leukocytes in the dermis, as described previously.^{13,21} On day 5 after infection, further epidermal thickening and increased leukocyte infiltration became evident in eczematous mice, and worsening of these changes and sloughing of the epidermis were observed on day 7 (Fig 2). However, none of these changes were seen in the majority of HSV1-infected normal mice. Only much milder changes were seen in about 20% of those mice (Fig 1, C). These results indicate that eczematous mice are more susceptible to HSV1 infection than normal mice. Consistent with this view, an mRNA coding for HSV1 *pol* was detected in the skin area of HSV1 inoculation on day 2 in eczematous but not normal mice (Fig 1, D). HSV1 *pol* expression peaked in eczematous mice on day 4 and returned to baseline by day 7. A major defect in the skin of patients with AD is a reduced epidermal skin barrier,^{1,2} which might be a predisposing factor for virus susceptibility.⁶ We confirmed increased TEWL in lesional skin of eczematous mice compared with normal mice (Fig 1, E).

Cytokine gene expression in our EH model

Gene expression patterns were shown to be similar between human AD skin and *D. farinae*/SEB-induced mouse skin lesions, including expression of genes related to immune responses.²¹ As shown previously,^{13,14} eczematous mice had increases in levels of T_H2 (IL-5, IL-9, and IL-10), T_H1 (IFN- γ), T_H17 (IL-17A, IL-17F, and IL-23), and proinflammatory (IL-1 α , IL-1 β , IL-6, IL-33, and TNF) cytokines compared with those seen in normal mice (Fig 3 and see Fig E1 in this article's Online Repository at www.jacionline.org). HSV1 infection induced T_H1 (IFN- γ) and T_H2 (IL-4, IL-9, and IL-10) cytokines in normal mice on day 2, but expression of these cytokine genes was reduced by day 4 (Fig 3 and see Fig E1). In contrast, expression of T_H1 and T_H2 cytokines was not increased further by HSV1 infection in eczematous mice. Expression of proinflammatory cytokines (IL-6, IL-1 α , IL-1 β , IL-33, and TNF) was also induced by HSV1 infection in normal mice and was further increased or sustained at high levels in eczematous mice. Some type I interferons (IFN- β 1 and IFN- α 7) were also induced in both normal and eczematous mice on HSV1 infection (Fig 3 and see Fig E1). A recent report showed that induction of mRNA and protein of type III interferon (IL-29) was lower among HSV1-infected patients with severe AD than among healthy subjects or patients with milder AD.²² We found that mRNA of type III interferons (IL-28A and IL-28B) was increased after HSV1 infection in normal mice, but induction was lower in eczematous mice (Fig 3).

Interestingly, clustering analysis revealed delayed upregulation of genes related to innate immune receptors, NK cells, and interferon responses in eczematous mice compared with normal mice (see Fig E2 in this article's Online Repository at www.jacionline.org). Collectively, the results are consistent with severe skin inflammation in HSV1-infected eczematous mice.

Reduced NK cell activity is responsible for early high EH scores on HSV1 infection

Our previous study showed that NK cell numbers are higher in eczematous lesions than in normal skin but NK cell activity is

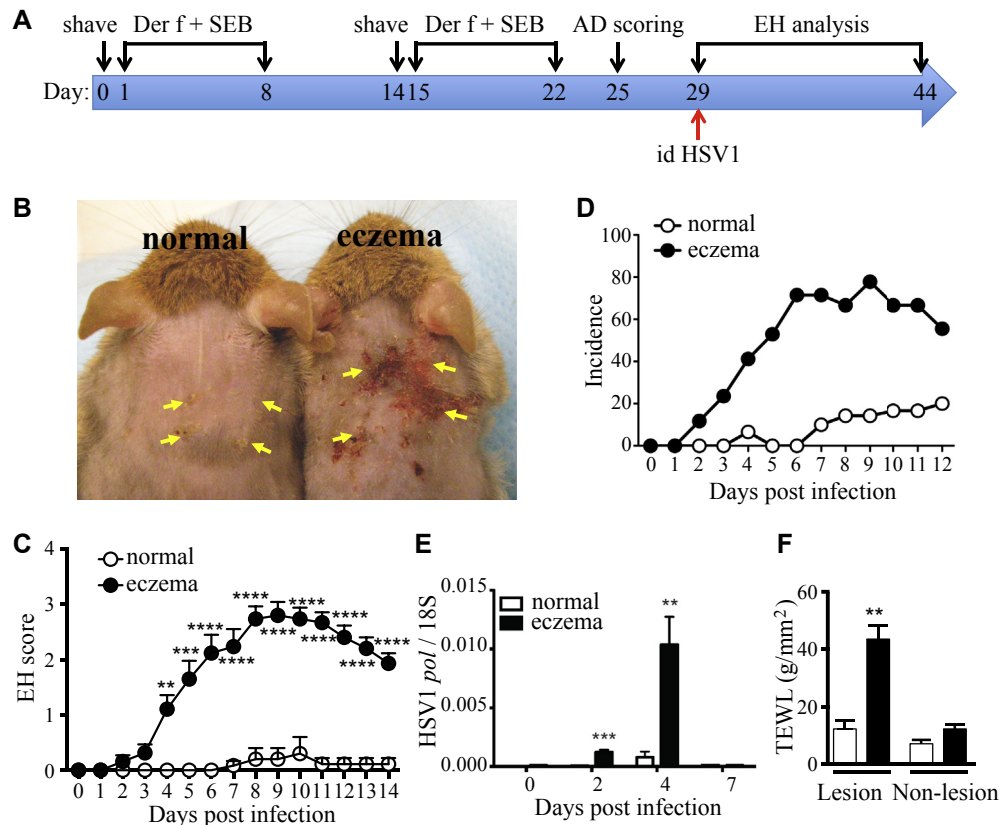


FIG 1. Clinical features of our EH model. AD-like skin lesions were induced by *D farinae* (Der f) and SEB. Then mice were infected with HSV1-McKrae. **A**, Scheme of experimental procedures. **B**, Severe lesions with bleeding and exudate on day 7 after infection in eczematous mice are shown along with a normal control mouse. **C**, EH scores in a representative experiment using 4 mice each. Similar results were obtained in at least 15 experiments. **D**, Accumulated time-course data with 15 normal and 17 eczematous mice. **E**, Viral titers. **F**, TEWL was assessed in lesional (Lesion) or anatomically identical normal skin or a distant area (Non-lesion) 3 days after the second *D farinae*/SEB treatment ended (before viral infection). Data represent means $\pm$ SEMs. ** $P < .01$, *** $P < .001$, and **** $P < .0001$, Student *t* test.

reduced by increased IL-17A expression in eczematous mice.¹⁴ These previous observations were supported by our microarray and qPCR analyses (Fig 3 and see Fig E2). We also confirmed that NK cell numbers in lesional skin of eczematous mice are not lower than those in normal mice before and after HSV infection (see Fig E3 in this article's Online Repository at www.jacionline.org). Given the importance of NK cell activity in the early defense against viral infection,^{23,24} we evaluated splenic NK cells in HSV1-infected eczematous mice. The maturation of mouse NK cells proceeds in 4 stages: CD11b^{low}CD27^{low} $\rightarrow$ CD11b^{low}CD27^{high} $\rightarrow$ CD11b^{high}CD27^{high} $\rightarrow$ CD11b^{high}CD27^{low}.²⁵ Because fully mature NK cells expressing CD43 are cytolytic and can produce IFN- γ after stimulation,^{26,27} we first enumerated splenic CD43^{hi} NK cells. As shown in Fig 4, A, and Fig E4 in this article's Online Repository at www.jacionline.org, there were no differences in mature NK cell proportions and CD43 expression before viral infection between eczematous and normal mice. Furthermore, the maturity of NK cells, as evaluated by expression of CD11b and CD27, was similar in normal and eczematous mice (Fig 4, B). Importantly, however, proportions of granzyme B⁺ NK cells and their granzyme B levels (as measured by using mean fluorescence intensity) were strongly reduced in HSV1-infected eczematous mice on day 2 after infection compared with those in

HSV1-infected normal mice (Fig 4, C and D, and data not shown). Similar results were observed on proportions of IFN- γ ⁺ NK cells and perforin-positive NK cells (Fig 4, D). These results indicate that NK cells in HSV1-infected eczematous mice were equipped with lower expression of effector molecules compared with those in the corresponding normal mice.

NK cells were depleted with anti-asialo GM1 antibody before HSV1 infection to investigate the role of NK cell activity in the development of HSV1-induced skin lesions. As previously shown,¹⁴ NK cells were depleted (data not shown). NK cell depletion in normal mice caused skin inflammation on HSV1 infection, although it did not cause a significant change in already high EH scores in eczematous mice (data not shown). However, because asialo GM1 is expressed in cells other than NK cells, anti-asialo GM1 antibody affects T cells and some myeloid cells.²⁸⁻³¹ Therefore we used anti-NK1.1 mAb to deplete NK cells more specifically. Similar to anti-asialo GM1 treatment, NK cell depletion caused skin inflammation in normal mice on HSV1 infection (Fig 5, A and B). Consistent with this, viral titers were increased on NK cell depletion (see Fig E5 in this article's Online Repository at www.jacionline.org). Second, NK cell depletion did not enhance already high EH scores in eczematous mice. To complement the depletion data, we performed adoptive transfer experiments. NK cell transfer to eczematous mice 1 day before

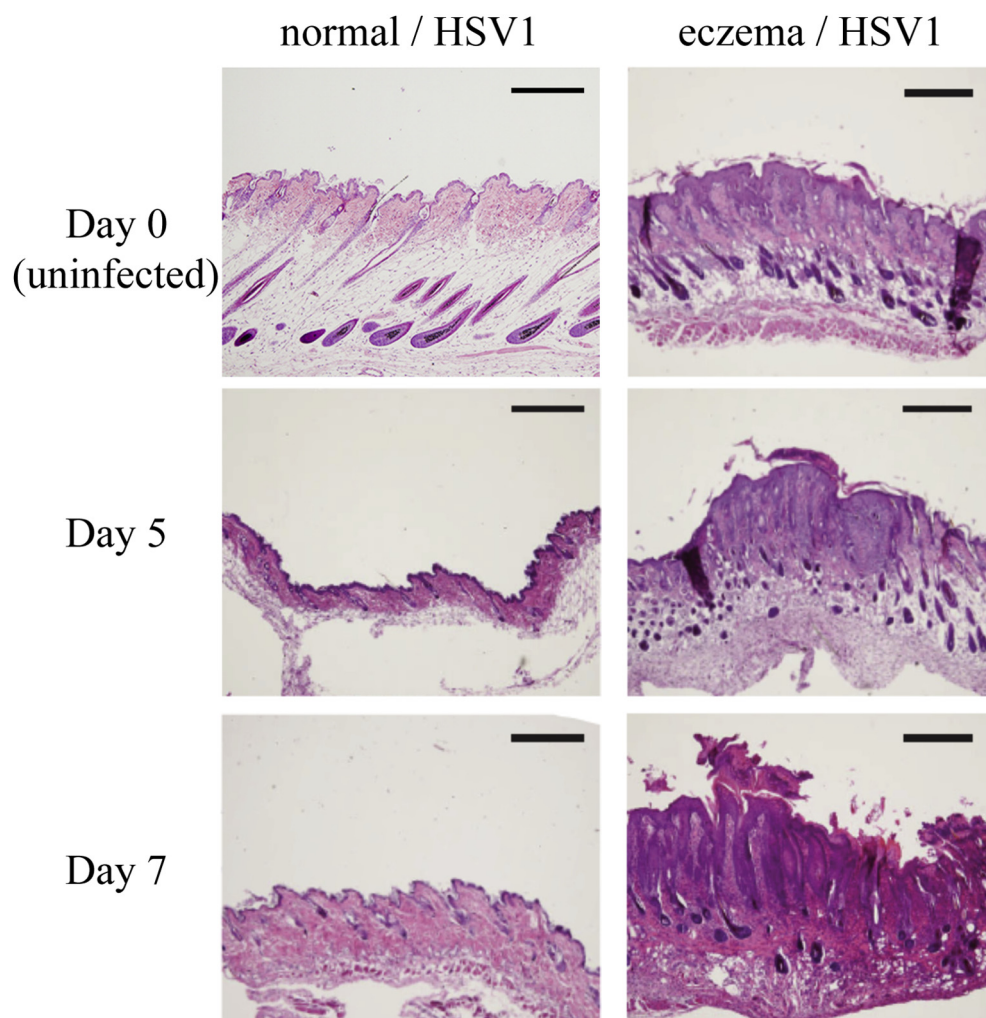


FIG 2. Histology of skin lesions in HSV1-infected eczematous mice. Hematoxylin and eosin–stained skin lesions before (day 0) and 5 and 7 days after HSV1 infection. Results are representative of 3 experiments.

viral infection reduced HSV1-induced EH scores at early time points (Fig 5, B and D). These results suggest that the reduced NK cell activity is responsible for HSV1-induced higher EH scores in eczematous mice.

Recently, NK cell activity was shown to be regulated by type III IFN- λ .³² IL-29 (IFN- λ 1) is not expressed in mice, and IL-28A (IFN- λ 2) and IL-28B (IFN- λ 3) proteins are 97% identical. IL-28A and IL-29 inhibited HSV1 replication in human neuronal cells.³³ In light of the reduced tendency for *Il28a* and *Il28b* mRNAs in HSV1-infected eczematous mice compared with normal mice (Fig 3), we tested whether these type III interferons play any role in the defense against HSV1-induced severe skin lesions. To this end, eczematous mice were treated with neutralizing anti-IL-28B mAb or recombinant IL-28A before and during HSV1 inoculation. In this experiment a lower titer of HSV1 was used to enable the effect of IL-28 blockade to be easily seen. IL-28 neutralization resulted in the development of more severe lesions than without IL-28 neutralization (see Fig E6 in this article's Online Repository at www.jacionline.org). On the other hand, mice injected with recombinant IL-28A did not show severe skin lesions. These results suggest that low levels of type III interferons contribute to lower NK cell activity.

CD8 T-cell and antibody responses against HSV1 appear comparable in normal and eczematous mice

Given the importance of cytotoxic T cells in the host defense against HSV infection,^{34,35} we examined CD8 T cells expressing killing mediators. On day 7 after infection, there were no significant differences in the proportions of CD8 T cells expressing granzyme B, perforin, or IFN- γ between normal and eczematous mice (see Fig E7 in this article's Online Repository at www.jacionline.org).

We also investigated humoral responses to HSV1 infection in eczematous mice. ELISA showed that there were no significant differences in IgG anti-HSV1 gD titers on days 7 and 14 after infection between normal and eczematous mice (Fig 6, A). No significant differences were found in total IgG levels in the 2 types of mice (Fig 6, B). T-cell help by T_{FH} cells is essential for antibody responses in germinal centers.³⁶ Therefore we quantified T_{FH} cells.³⁷ We found higher numbers of T_{FH} cells in skin-draining lymph nodes before and after HSV1 infection in eczematous mice than in normal mice (see Fig E8 in this article's Online Repository at www.jacionline.org). These results indicate that antiviral or general humoral responses are not impaired in eczematous mice, similar to what is seen in vaccinia virus–infected eczematous mice.¹⁴

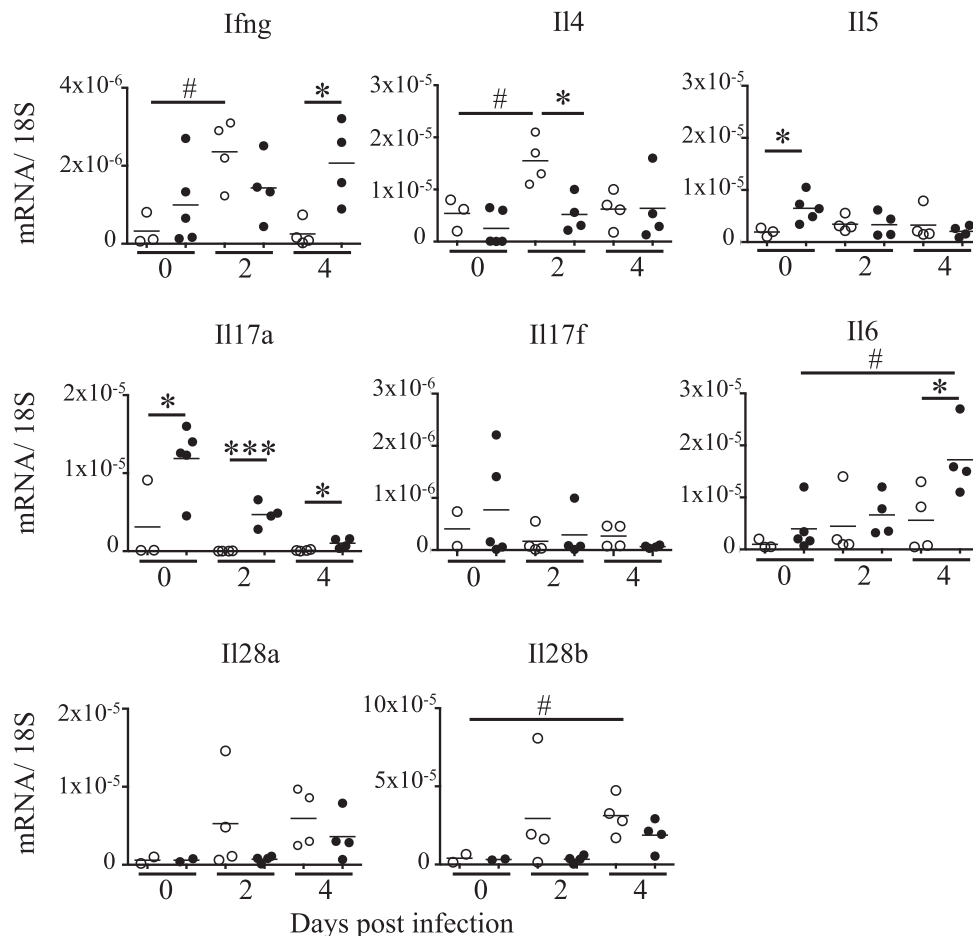


FIG 3. Expression of cytokine genes in our EH model. AD-like skin lesions were induced by *D farinae* and SEB in NC/Nga mice. RNAs from lesional or anatomically identical normal skin portions from 2 to 5 mice were used to prepare cDNAs, and qPCR analysis was performed. Results shown here are consistent with those obtained by using the microarray analysis shown in Fig E1. Open circles, Normal skin; filled circles, eczematous skin. * $P < .05$ and *** $P < .001$ versus normal mice and # $P < .05$ versus day 0, Student *t* test.

Treg cells were more abundant in eczematous mice

In an EC-OVA model of AD, Treg cells were shown to be essential suppressors of AD-like skin inflammation.³⁸ By showing an increased frequency of Treg cells with skin-homing and activated phenotypes in patients with AD and EH, a recent study suggested that expansions of functional Treg cells initially required for ameliorating excessive inflammation could contribute to the initiation and progression of HSV reactions, causing EH.⁸ Therefore we enumerated CD4⁺CD62L⁺CD44⁺Foxp3⁺ Treg cells in skin-draining lymph nodes. Although total splenic CD4⁺ cells were comparable in normal and eczematous mice (data not shown), percentages of Treg cells in CD4⁺ cells in the draining lymph nodes were slightly lower in eczematous mice than in normal mice before HSV1 infection, but absolute numbers of Treg cells were more abundant in eczematous mice (Fig 7, Day 0), which is similar to the EC-OVA model.³⁸ Seven and 10 days after infection, which were just before the resolution phase (Fig 1, B and C), Treg cells were still more abundant in eczematous than in normal mice (Fig 7). We confirmed that Treg cell functions are comparable in normal and eczematous mice (see Fig E9 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Impaired skin barrier function is an underlying factor for AD, as well as increased susceptibility to HSV1 infection. In this study we showed that reduced NK cell activity (not number) plays a critical role in the HSV1-induced development of severe skin lesions in eczematous mice. The role of NK cells in defense against HSV1-induced skin lesions in our EH model is similar to that found in our eczema vaccinatum model.¹⁴ NK cell depletion and reconstitution were performed similarly in both eczema vaccinatum and EH models. In the previous article we showed that IL-17A reduced NK cell activity before viral infection. Because the procedures to induce AD-like skin inflammation are shared by the 2 models, our results in the current study suggest that high IL-17A expression should cause reduced NK cell activity. In addition to IL-17A, IL-6 and IL-10 in eczematous mice also seem to contribute to the reduced NK cell activity, with each inhibiting this activity.¹⁴ On the other hand, IL-28 might be an important factor for the defense against HSV1-induced EH. Thus low levels of type III interferons might contribute to lower NK cell activity. Consistent with these animal studies, it was reported that patients with AD have NK cell defects.^{9,39,40} Therefore the defective NK cell activity in patients

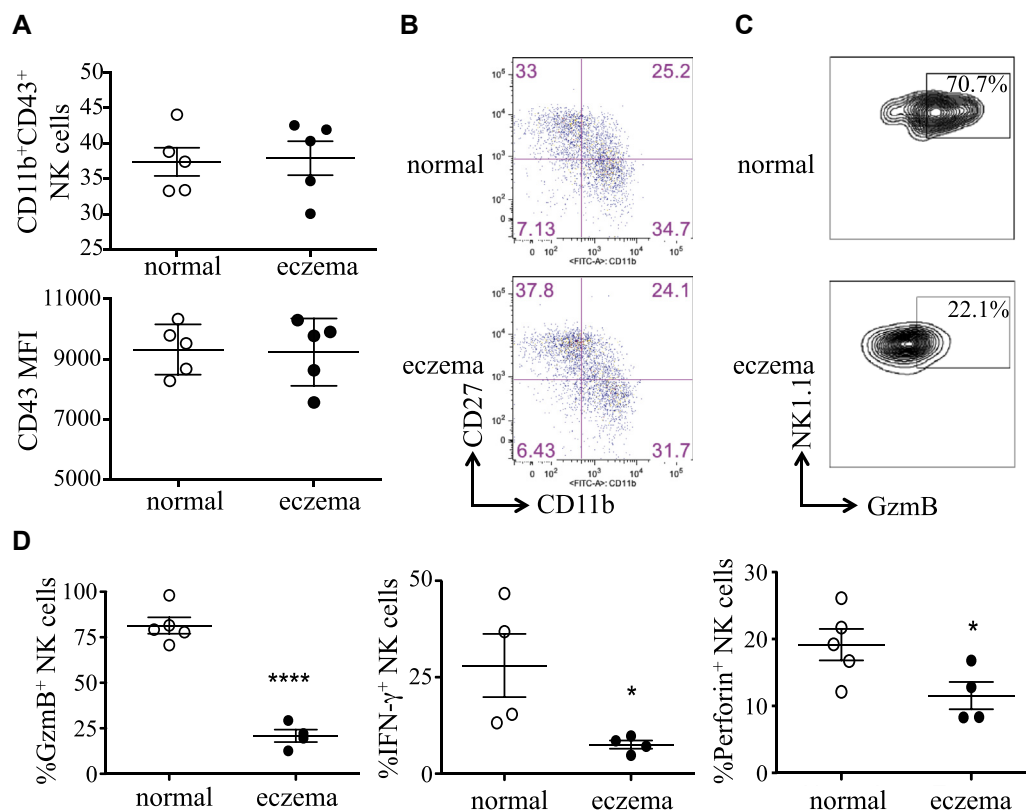


FIG 4. NK cells have lower levels of granzyme B, perforin, and IFN- γ in eczematous mice. Flow cytometric analysis of NK cells is shown, and results are representative of 3 experiments. **A** and **D**, Quantification of flow cytometric data. **B** and **C**, Representative results of flow cytometry. *MFI*, Mean fluorescence intensity. Data represent means $\pm$ SEMs. * $P < .05$ and **** $P < .0001$, Student *t* test.

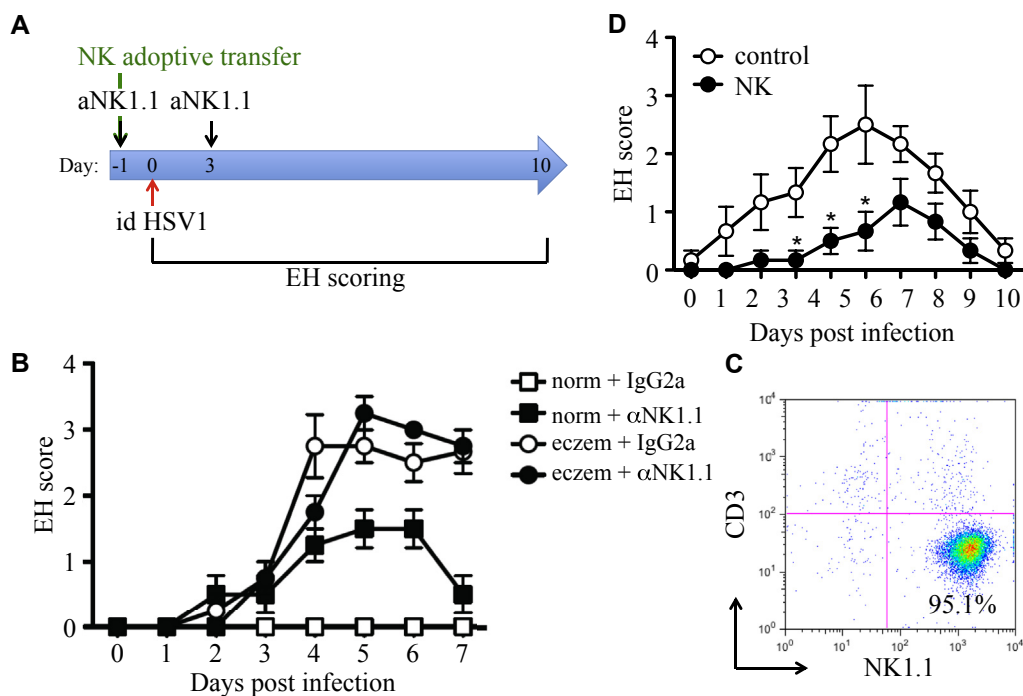


FIG 5. NK cells play a critical role in protecting mice from HSV1-induced early skin lesions. NK cells were depleted by intravenous injections of anti-NK1.1 mAb on day -1 before infection and day 3 after infection or adoptively transferred on day -1. Control mice were treated with control IgG_{2a}. **A**, Experimental procedures. AD induction is omitted from the scheme. **B**, EH scores on NK cell depletion. **C** and **D**, NK cells (>95% pure; Fig 5, C) were adoptively transferred to the AD-induced NC/Nga mice 24 hours before HSV1 infection. * $P < .05$. N = 4 (Fig 5, B) and n = 5 (Fig 5, D) for each cohort. Results are representative of 3 experiments.

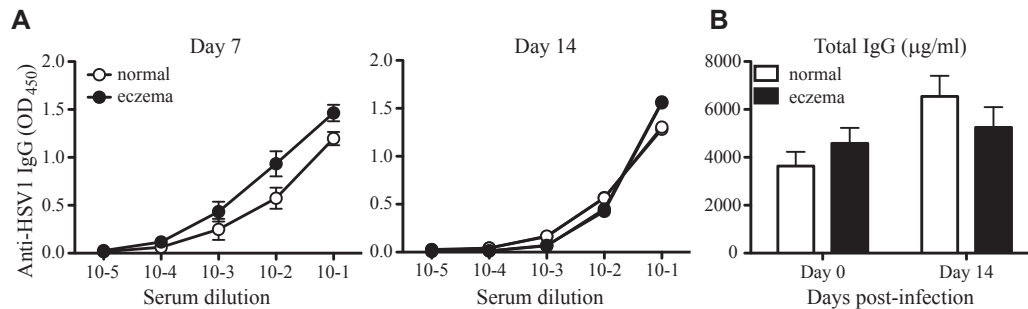


FIG 6. Anti-HSV1 IgG antibody responses in eczematous and normal mice. ELISA was performed on mouse sera by using HSV1 gD-Fc fusion protein as a capturing agent for anti-HSV1 IgG (A) or total IgG (B). N = 5 each for normal and eczematous mice. Results are representative of 3 experiments.

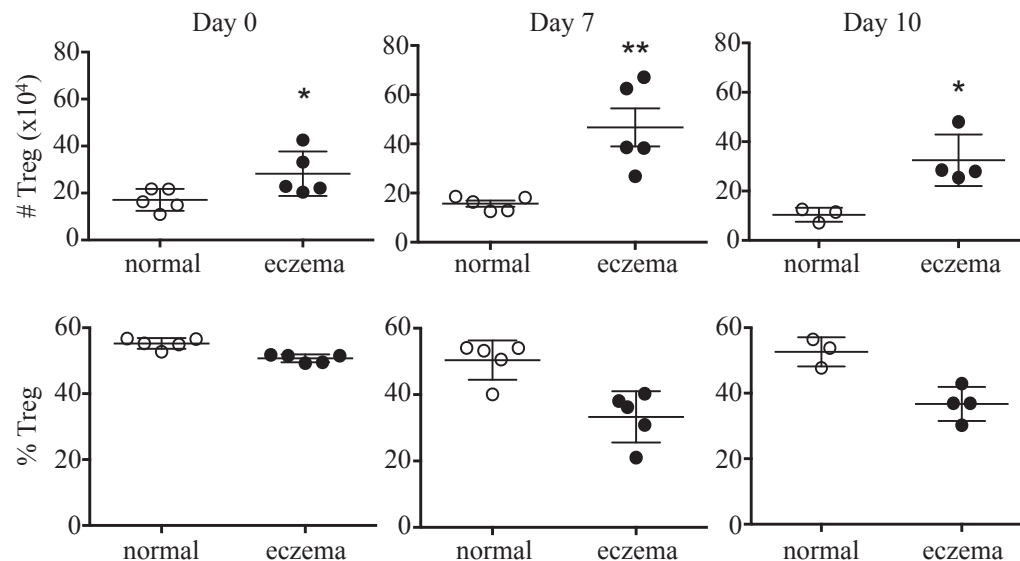


FIG 7. Increased Treg cell numbers in eczematous mice. Foxp3⁺ Treg cells in skin-draining lymph nodes were enumerated by means of flow cytometry. **P* < .05 and ***P* < .001. Results are representative of 3 experiments.

with AD seems to be a common predisposing factor for increased susceptibility to different viruses.

Severe skin lesions could be induced by HSV1-McKrae but not strain HSV1-KOS in eczematous mice. Numerous small vesicles with exudates appeared over the back skin on days 4 to 7 after infection of KOS and started to subside by day 12 (see Fig E10 in this article's Online Repository at www.jacionline.org). However, inoculation (10² to 10⁶ plaque-forming units per inoculation) of KOS did not induce skin lesions consistently. KOS is frequently used to investigate HSV-1 gene function and pathogenesis. KOS is less virulent than other HSV1 strains, such as McKrae and 17.⁴¹ Comparing the genome of strain KOS with that of strain 17 revealed that the genomes differ by at least 1024 single nucleotide polymorphisms and 172 insertions/deletions,⁴² and comparing McKrae's genome with that of strain 17 revealed that the genomes differ by at least 752 single nucleotide polymorphisms and 86 insertion/deletions.⁴³ Some of these differences might be related to the weak and strong virulence of the Kos and McKrae strains, respectively. However, in our literature search we did not find whether the HSV found in patients with EH is particularly virulent.

Transcriptomic analysis showed complex patterns in gene expression during HSV1 infection in eczematous versus normal mice. Consistent with the role of NK cells in defense against viruses, some genes related to innate immune receptors and NK cells exhibited delayed upregulation in eczematous mice compared with normal mice. The innate immune response to viral infection is linked to adaptive immunity. However, no differences were found in anti-HSV1 antibody responses or total IgG levels in the 2 types of mice. Both T_{FH} and Treg cells were more abundant in eczematous than normal mice, but the differences in T_{FH} cell numbers became insignificant 7 and 10 days after HSV1 infection. Thus adaptive immunity might not be different in eczematous and normal mice.

Forkhead box protein 3 (Foxp3) functions as a master transcriptional regulator in the development and function of Treg cells.^{44,45} Mutations in the *Foxp3* gene in immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome^{46,47} and scurfy mice⁴⁸ cause multiple allergies, including eczema, as well as overproliferation of CD4⁺ T cells, extensive multiorgan infiltration, and increase in levels of numerous cytokines. In the EC-OVA model numbers of Foxp3⁺ Treg cells were increased

in the allergen-exposed skin area and draining lymph nodes, and depletion of Treg cells led to exacerbated skin inflammation and increased serum IgE levels.³⁸ In line with this and our current data, several studies showed increased Treg cell numbers in patients with AD.⁴⁹⁻⁵² However, others found decreased numbers of Treg cells in the peripheral blood of patients with AD.⁵³ Shiohara et al⁵⁴ suggested an imbalance between antiviral immune responses and Treg cells as a potential mechanism for EH. In their scenario expansions of Treg cells would be required initially for preventing the excessive inflammation caused by AD, and the expansions could in turn contribute to HSV reactivation, resulting in initiation and progression of EH. Thus it is warranted to study the contribution of Treg cells to EH in the future.

In sum, we established a murine model of EH in this study. Using this model, we demonstrated the critical role of defective NK cell activities in the development of HSV1-induced severe skin lesions in eczematous mice. Given the low NK cell activities in patients with AD, our results can explain an increased susceptibility of patients with AD to HSV and point to the potential utility of testing NK cell activity in patients with AD to predict high risk of subsequent EH.

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Key messages

- We established a murine model of EH in this study.
- We demonstrated a critical role of defective NK activities in HSV1-induced severe skin lesions in eczematous mice.

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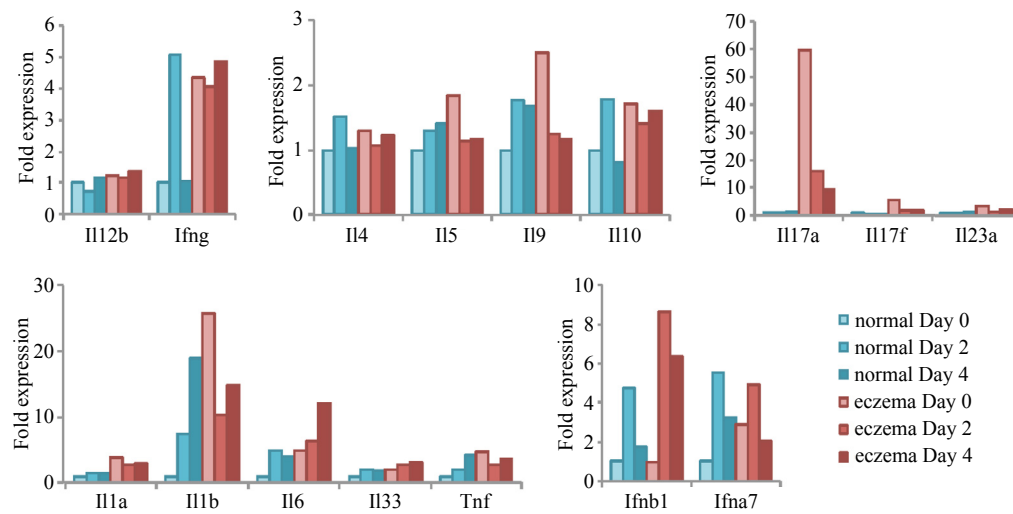
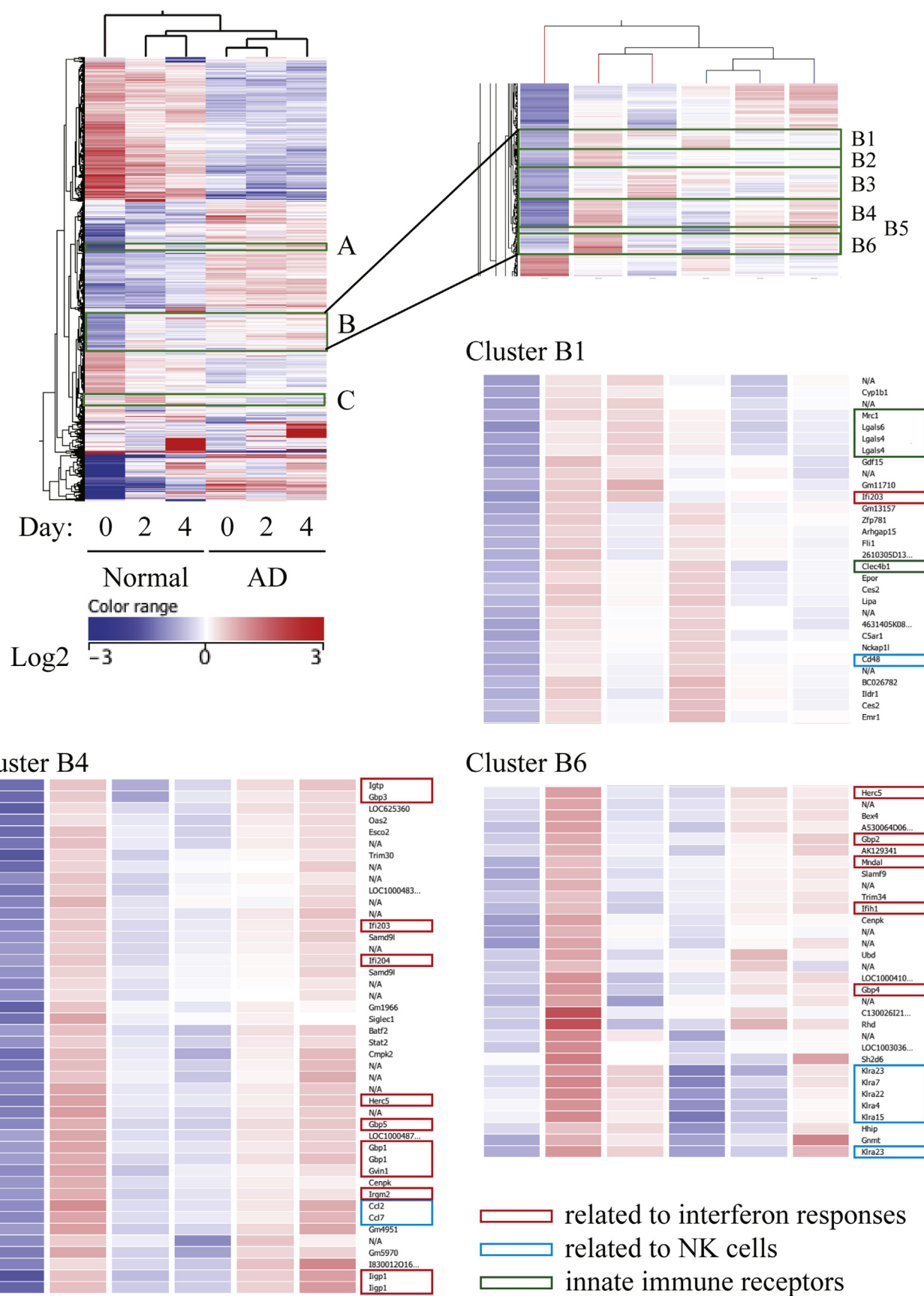


FIG E1. Expression of cytokines before and after HSV1 infection. RNAs from lesional or anatomically identical normal skin portions from 3 to 5 mice were combined and analyzed by using a microarray. Results shown here are consistent with qPCR analysis, as shown in [Fig 3](#).



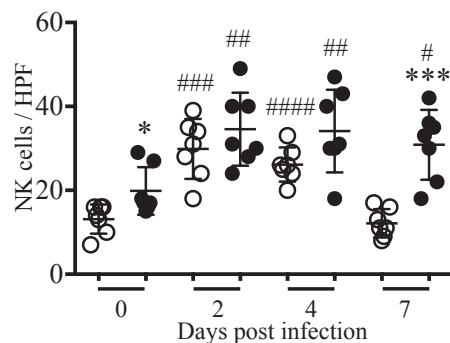


FIG E3. NK cells in normal and eczematous skin. Back skin of normal or eczematous mice was cut out before or 2 days after HSV1 infection. The frozen sections were stained with anti-Ly49G2 for immunohistochemical analysis. *Open circles*, Normal skin; *solid circles*, eczematous skin. *HPF*, High-power field. * $P < .05$ and *** $P < .001$ versus normal skin and # $P < .05$, ## $P < .01$, ### $P < .001$, and #### $P < .0001$ versus day 0, Student t test.

FIG E2. Clustering analysis of skin-expressed genes. Of 55,821 probes, 26,288 genes were greater than 100 in raw intensity in at least 1 condition. Among them, more than 2-fold changes were detected by 7750 probes after infection or between normal and eczematous mice. These 7750 probes were subjected to the hierarchical clustering. We detected 3 clusters of genes that seem to be related to the EH phenotype. Cluster A contains genes upregulated by inflammation caused by either infection or AD induction. Cluster B comprises some subclusters, most of which are composed of the genes with expression induced by infection, but the induction was impaired in AD-induced skin. In subclusters B1, B4, and B6, genes related to NK cells, interferon responses, and innate immune receptors were found. Cluster C includes genes upregulated by infection but not so much by AD induction.

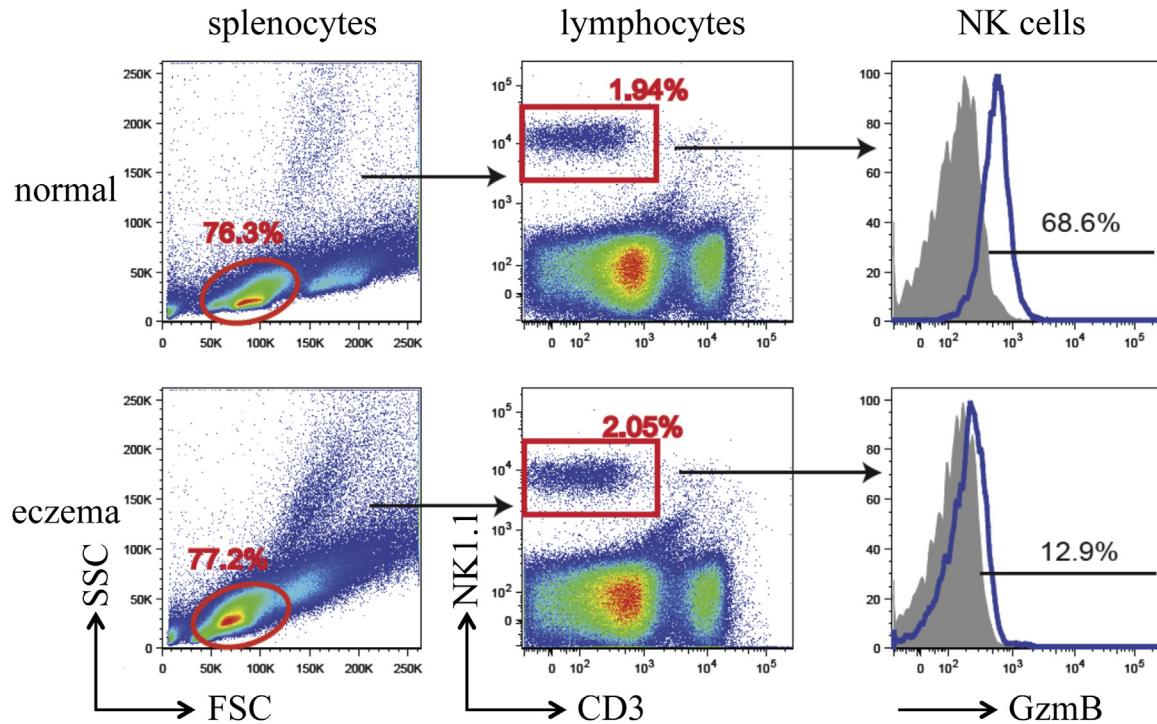


FIG E4. Reduced expression of granzyme B (*GzmB*) in NK cells in eczematous mice. Spleen cells were stained for CD3 and NK1.1 for flow cytometry. The gating strategy is shown from left to right. *FSC*, Forward scatter; *SSC*, side scatter.

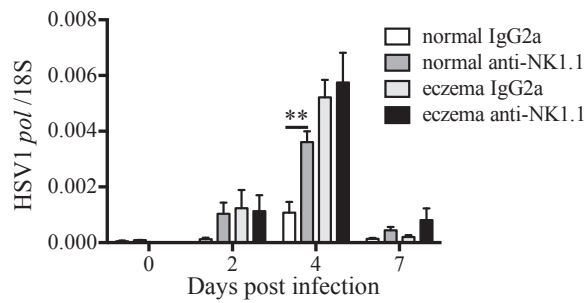


FIG E5. Depletion of NK cells increases replication of HSV1 in infected skin on day 4 after infection. NK cells were depleted by anti-NK1.1 antibody. HSV1 viral load was quantified by using qPCR. Data are representative of 2 experiments with 4 mice per each group. $**P < .001$, Student *t* test.

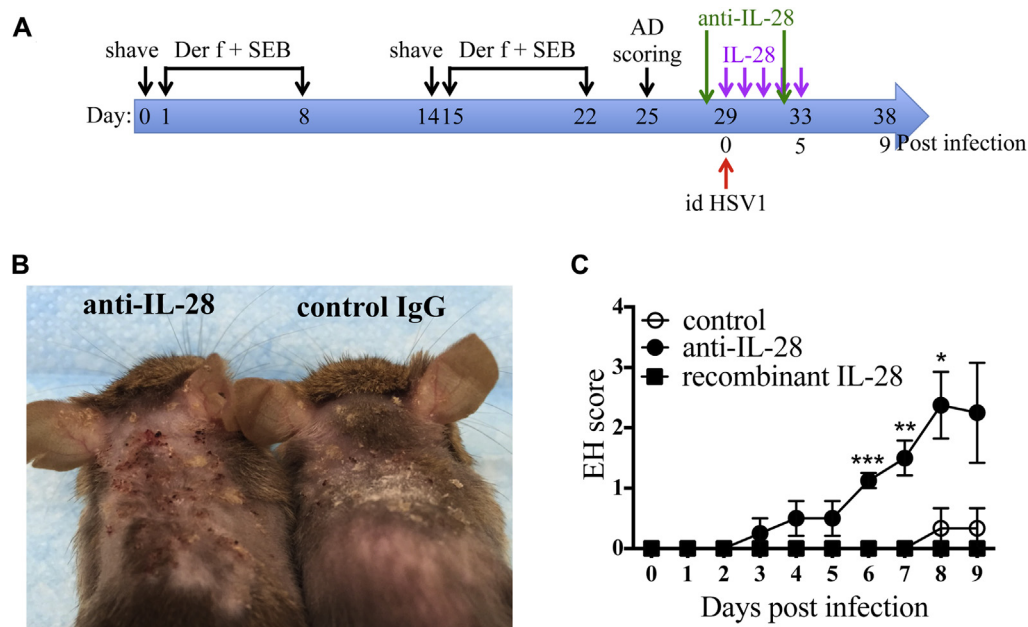


FIG E6. IL-28A and IL-28B might play a protective role in defense against HSV1-induced EH-like skin lesions. AD-like skin lesions were induced by *D farinae* (*Der f*) and SEB. Anti-mouse IL-28B (100 μ g; InvivoGen, San Diego, Calif) or control mouse IgG1 was intraperitoneally injected 1 day before and 4 days after HSV1 infection to deplete IL-28. We injected intraperitoneally 0.4 mg/kg body weight of recombinant mouse IL-28A (mouse IFN- λ 2, PeproTech) or PBS daily on days 0, 1, 2, 3, 4, and 5 after HSV-1 infection. Mice were infected with HSV1-McKrae (2×10^3 plaque-forming units per site on the 4 sites) on day 0 (day 29 in the scheme). **A**, Scheme of experimental procedures. **B**, Severe lesions with bleeding and exudate on day 7 after infection in anti-IL-28B-treated versus control mouse IgG₁-treated eczematous mice are shown. **C**, EH scores in a representative experiment with 4 mice each. Data represent means $\pm$ SEMs. * $P < .05$, ** $P < .001$, and *** $P < .0001$, Student *t* test.

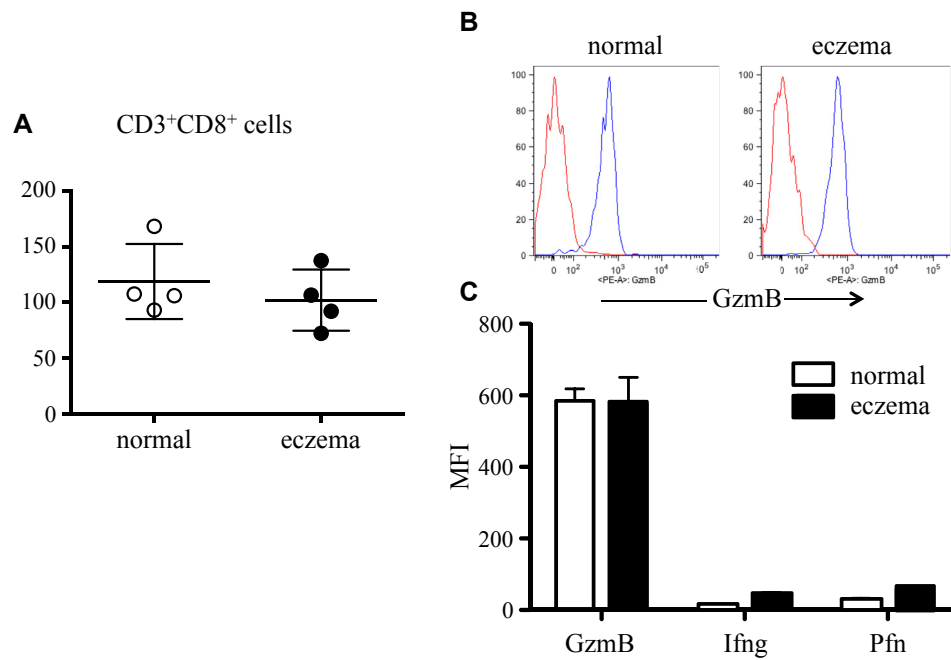


FIG E7. Expression of killing mediators in cytotoxic T lymphocytes is similar in normal and eczematous mice. **A**, CD3⁺CD8⁺ cells in spleens were quantified by using flow cytometry. **B**, Expression of granzyme B (*GzmB*) in CD3⁺CD8⁺ cells was evaluated by means of flow cytometry. Results are representative of 2 experiments. **C**, Quantification of expression of granzyme B, IFN- γ , and perforin (*Pfn*) in NK cells, as evaluated by using flow cytometry. *MFI*, Mean fluorescence intensity.

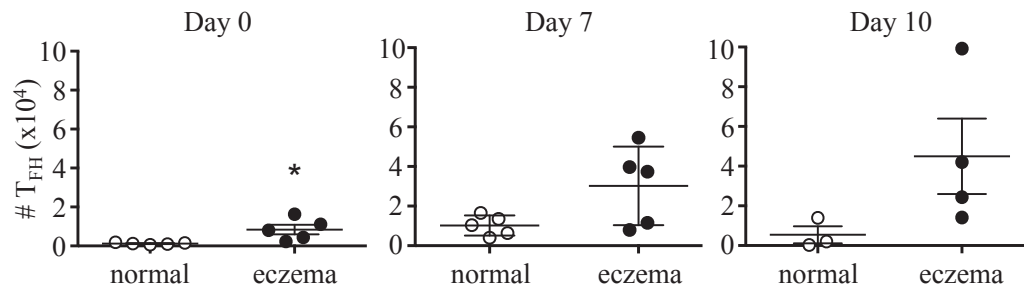


FIG E8. Increased numbers of T_{FH} cells are found in skin-draining lymph nodes of eczematous mice. $CD4^+CD62L^-CD44^+CXCR5^+Bcl6^+T_{FH}$ cells were enumerated by using flow cytometry. * $P < .05$.

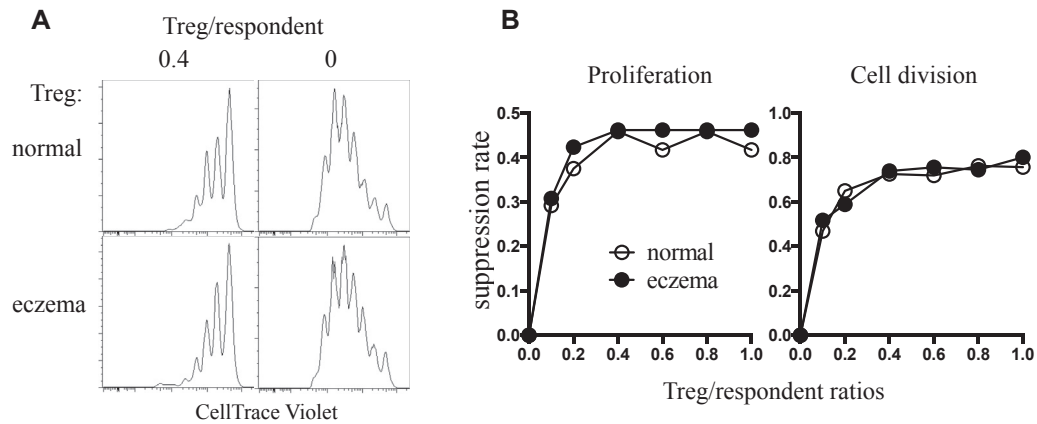


FIG E9. Treg cells from eczematous mice are functionally normal. Treg cells were sorted as $CD4^{+}CD25^{high}$ cells from spleens of eczematous and normal mice. CellTrace Violet-labeled responder cells ($CD4^{+}CD45RB^{high}$) were cultured with serially diluted Treg cells from eczematous or normal mice with antigen-presenting cells and stimulated with soluble anti-CD3 ϵ antibody. Treg cell function on proliferation of responder cells was analyzed by using fluorescence-activated cell sorting. **A**, Division of responder cells detected by using fluorescence-activated cell sorting. **B**, Suppression rate of proliferation index and division index were calculated and plotted.

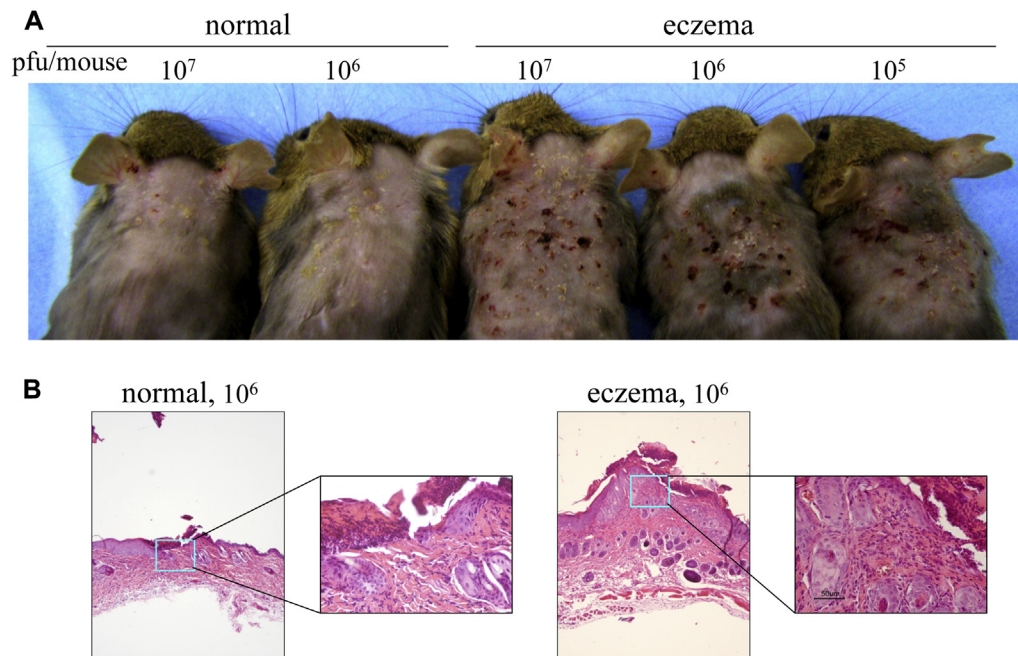


FIG E10. HSV1 Kos strain induced skin lesions with bleeding and exudate on eczematous mice but not on normal skin mice. Different doses of HSV1 Kos were inoculated on the lesional or same anatomic site of normal or eczematous mice, followed by scarification with a 26-gauge needle. **A**, Skin lesions with bleeding on day 7 after infection in eczematous mice are shown with normal control mice. **B**, Back skin was excised on day 7 after infection, fixed, paraffin embedded, and stained with hematoxylin and eosin. Data are representative of 4 experiments, with 4 to 8 mice per each group each time.