Inhibition of NK cell activity by IL-17 allows vaccinia virus to induce severe skin lesions in a mouse model of eczema vaccinatum

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Threats of bioterrorism have renewed efforts to better understand poxvirus pathogenesis and to develop a safer vaccine against smallpox. Individuals with atopic dermatitis are excluded from smallpox vaccination because of their propensity to develop eczema vaccinatum, a disseminated vaccinia virus (VACV) infection. To study the underlying mechanism of the vulnerability of atopic dermatitis patients to VACV infection, we developed a mouse model of eczema vaccinatum. Virus infection of eczematous skin induced severe primary erosive skin lesions, but not in the skin of healthy mice. Eczematous mice exhibited lower natural killer (NK) cell activity but similar cytotoxic T lymphocyte activity and humoral immune responses. The role of NK cells in controlling VACV-induced skin lesions was demonstrated by experiments depleting or transferring NK cells. The proinflammatory cytokine interleukin (IL)-17 reduced NK cell activity in mice with preexisting dermatitis. Given low NK cell activities and increased IL-17 expression in atopic dermatitis patients, these results can explain the increased susceptibility of atopic dermatitis patients to eczema vaccinatum.

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The smallpox vaccine consists of live vaccinia virus (VACV) and is considered the gold standard of vaccines, as it has led to the complete eradication of a lethal infectious disease from the human population. Recent fears that smallpox might be deliberately released in an act of bioterrorism have led to renewed efforts to better understand the disease mechanism and to develop a safer vaccine. Approximately 50% of US residents were born after the regular smallpox vaccination was discontinued in 1972. Thus, these unimmunized people are vulnerable to smallpox. The population landscape is very different between now and 36 yr ago, with two-to-three times more frequent incidence of atopic dermatitis in the current population (1). Individuals with atopic dermatitis are excluded from smallpox vaccination because of their propensity to develop eczema vaccinatum, a disseminated vaccinia infection (2).

Atopic dermatitis is a chronic inflammatory skin disease (3). The etiology of this disease is multifactorial, and involves complex interactions between genetic and environmental factors. The skin in a preatopic dermatitis state has been postulated to have hypersensitivity to environmental triggers, resulting from a defective skin barrier that allows the penetration of allergens and microbial pathogens (4). The acute phase is characterized by eczematous skin lesions with an infiltration of Th2 cells. The chronic phase is characterized by lichenification of skin and an infiltration of Th1 cells. As recent studies have established IL-17– and IL-22–producing $CD4^+$ T cells as a distinct class of helper T cells (Th17), Th17 cells are also implicated in the acute but not the chronic phase (5, 6). Despite the progress in our understanding of atopic dermatitis pathogenesis (7) and immune responses to VACV (8), it is not understood why atopic dermatitis patients are susceptible to developing eczema vaccinatum (9).

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In this study, we have established a mouse model of eczema vaccinatum using a strain of mice that are prone to develop eczematous skin lesions, characterized their immune responses to VACV infection, and showed the importance of NK cells in early suppression of VACV-induced severe eczema vaccinatum–like skin lesions.

RESULTS AND DISCUSSION

We initially focused on establishing experimental conditions in which infection with VACV induces differential clinical outcomes between mice with and without eczematous skin lesions. Skin lesions were induced on the backs of dermatitis-prone NC/Nga mice (10) by epicutaneous treatment of shaved skin with a mite extract and staphylococcal enterotoxin B (SEB), as described previously (11). This treatment induced elevated serum IgE levels and eczematous skin lesions (Fig. 1 A) (11). Skin lesions with maculopapular rash started to appear on the infected site on day 2–3 after infection in eczematous mice and developed into severe skin erosion. The size of the primary lesion peaked at days 7–8 (Fig. 1, B and C), and the lesion began to subside by day 11. Unlike eczematous mice, most normal mice failed to develop skin lesions after VACV infection, and even when developed, their skin lesions were much milder (Fig. 1, B and C). Virus titers in the lesional skin of eczematous mice were 300–10,000 times higher than those of normal mice over an observation period of 14 d (Fig. 1 D). In erosive skin lesions of eczematous mice, epithelial layers were separated from the rest of the skin and more leukocytes infiltrated the diseased dermis (Fig. 2, A and B). Pock-like satellite lesions distant from inoculation sites were rarely seen (only 3 cases out of 230 eczematous mice and 0 out of 187 normal mice). Although weight loss was observed in a small number of both eczematous and normal mice, there was no correlation with skin conditions (unpublished data). Unlike the intradermal infection at eczematous skin lesions, intranasal infection or intradermal infection at distant normal skin sites failed to induce clinical conditions (e.g., weight loss, survival, and size of skin lesions) distinctly different between eczematous

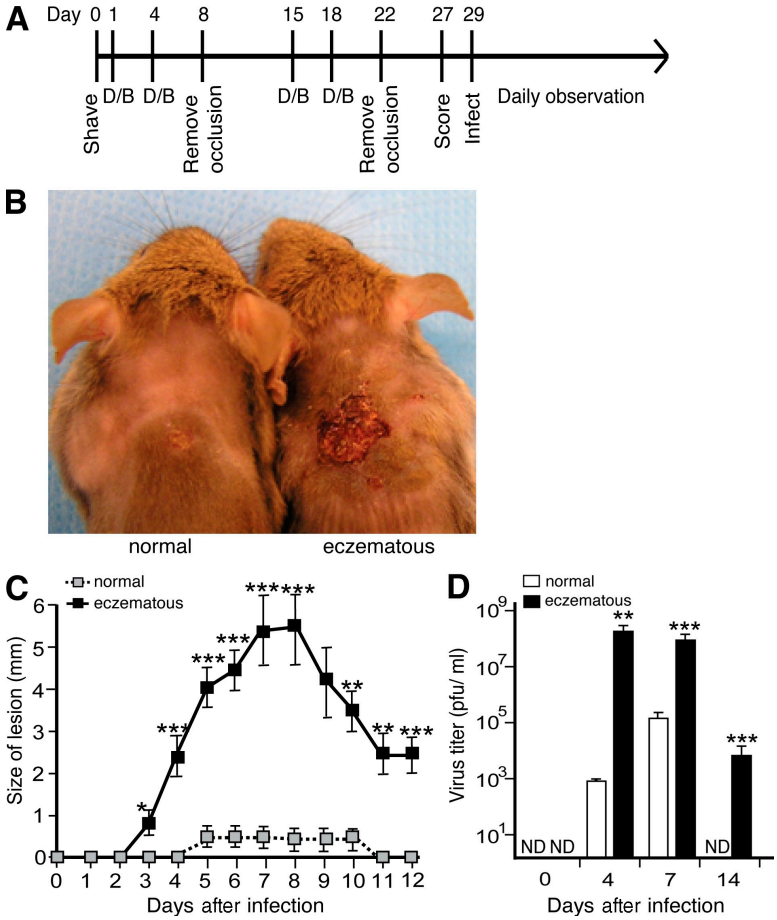

Figure 1. Induction of erosive primary skin lesions in VACV-infected eczematous mice. (A) Eczematous skin lesions were induced by repeated Der f/SEB (D/B) treatments, and mice with a clinical score of ≥8 were infected intradermally with VACV (eczematous group). A cohort (normal group) of mice with healthy skin was also infected at the same anatomical site. (B) Typical eczematous (right) and normal (left) mice are shown on day 6 after infection. (C) The size of erosive skin lesions. Shown is a representative of at least 15 experiments using 4–10 mice in each group. (D) Virus titers in the infected skin (n = 7 mice per group). Shown are results representative of four independent experiments. Data represent means and SEM values. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 versus normal mice. ND, not detected.

and normal mice (unpublished data). Unlike the Western Reserve strain used throughout this study, intradermal infection with the same dose of ACAM2000, the licensed vaccine cloned from Dryvax, caused much milder skin lesions compared with Western Reserve–induced skin lesions (unpublished data).

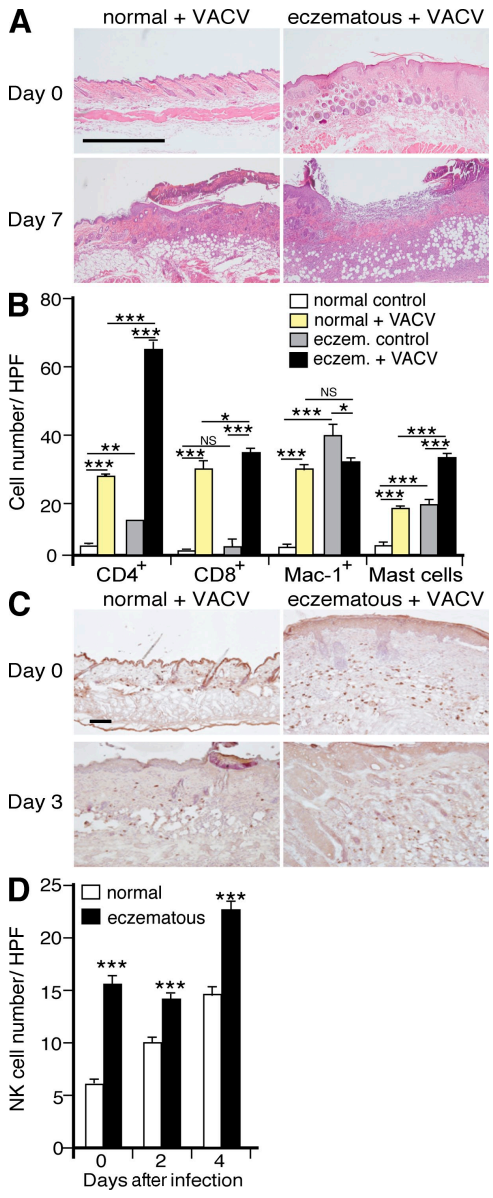

Figure 2. Histology of skin lesions before and after VACV infection. (A) Hematoxylin and eosin–stained skin tissues are shown for normal and eczematous mice before and 7 d after virus infection. Bar, 1 mm. (B) CD4+, CD8+, and Mac-1+ cells were stained by immunohistochemistry and mast cells were stained with toluidine blue. Data represent means and SEM values of cell numbers per high-power field (HPF; $n = 8$ each group). (C) Immunohistochemical staining of NK cells with anti-Ly49G2 (clone 4D11) mAb. Bar, 0.1 mm. (D) Ly49G2 (4D11)+ NK cells were enumerated with six mice per cohort. Shown are results representative of six independent experiments. Data represent means and SEM. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. NS, not significant.

Because of the importance of NK cells in rapid antiviral defense (12, 13), we quantified their numbers and activities. NK cells were more abundant in primary skin lesions in eczematous than normal mice (Fig. 2, C and D). Importantly, NK cell cytotoxic activity in the spleen was lower in eczematous mice on days 2 and 3 after infection (Fig. 3 A). We measured expression of molecules involved in NK killing activity by flow cytometry and found that the proportions of splenic NK cells expressing granzyme B, perforin, and IFN-γ were significantly lower in eczematous mice (Fig. 3 B).

IgM and IgG responses against VACV were similar between the eczematous and normal cohorts (Fig. S1 A). Consistent with this, IL-4 mRNA levels in lymph nodes were not reduced in eczematous mice for the initial 7 d after infection (Fig. S1 B). Killing activity of $CD8^+$ T cells and their expression of granzyme B, perforin, and IFN-γ in day 7 spleens did not show differences between the two cohorts (unpublished data). These results suggest that adaptive immunity does not play a major role in causing differential skin outcomes of VACV infection between the eczematous and normal mice, although these arms of immunity are critical in the control of virus infection in vaccinia-infected mice (8).

The role of NK cells in this eczema vaccinatum model was assessed by depletion studies. First, dermatitis was induced in NC/Nga mice. 1 d before infection and on d 3 after infection, mice were intravenously injected with anti–asialo GM1 (αAGM1) or control rabbit serum (NRS). Treatment with αAGM1 serum drastically reduced the numbers of $NK1.1^+$ cells in the spleens (73–89% reduced as evaluated by flow cytometry) and suppressed NK cell activity in spleens in day 3–infected normal mice (normal/αAGM1 group) compared with NRS-treated normal mice (normal/NRS group; Fig. S2). In contrast, αAGM1 treatment did not significantly reduce the already low NK cell activity in day 3–infected eczematous mice. Substantially higher virus titers were observed in lesional skins (Fig. 3 C) and lungs (not depicted) of αAGM1-treated normal mice than those of NRS-treated normal mice. Importantly, 14 out of 16 mice in the normal/αAGM1 group exhibited erythematous papules at the inoculation site by day 6, whereas only 1 out of 13 mice in the normal/NRS group developed such a lesion. Some normal/αAGM1 and eczematous/αAGM1 mice developed satellite lesions as well (Fig. 3 E). Eczematous mice developed larger erosive skin lesions at the site of virus inoculation than noneczematous mice (Fig. 3 D). These primary lesions in NK-depleted eczematous mice were significantly larger than lesions in control eczematous mice (Fig. 3 D). As αAGM1 treatment might affect other cell types besides NK cells (14), we performed a second experiment in which we depleted NK cells by administering anti-NK1.1 mAb. Results were similar to those with αAGM1 (Fig. S3).

To complement the NK depletion experiments, we performed adoptive transfer of NK cells to determine whether activated NK cells could rescue NC/Nga mice from eczema vaccinatum. NK cells were obtained by culturing splenocytes in IL-15 for 4 d. The cultured cells, composed of a >95% $CD3^-$ $NK1.1^+$ population (Fig. 3 F, inset), were intravenously

transferred to eczematous or normal mice. Transfer of NK cells either totally suppressed the development of erosive skin lesions or greatly reduced skin lesion sizes (Fig. 3 F). The activated NK cells also delayed the kinetics of lesion development in the subset of mice that eventually developed erosive skin lesions. Therefore, the NK depletion and transfer experiments demonstrate a critical role for NK cells in protecting mice from developing VACV-induced erosive skin lesions and satellite skin lesions in this NC/Nga mouse model of eczema vaccinatum.

NK cell function is under the control of various cytokines, including IL-6 and IL-10, which each inhibit NK cell activity. The proinflammatory cytokine IL-17 is produced by Th17 cells (15). In eczematous mice, real-time PCR analysis of splenocytes showed increased mRNA expression of IL-17A and the cytokines involved in Th17 development (IL-6, TGF-β, IL-21, and IL-23) and effector functions (IL-21 and IL-22) (Fig. 4 A). IL-17A and IL-6 mRNAs were also increased in lesional skins of uninfected eczematous mice, whereas IL-17A, IL-6, and IL-23 mRNAs were increased in draining lymph nodes of eczematous mice (Fig. 4 A). Consistent with these mRNA results, lymph nodes contained an increased number of Th17 cells in eczematous mice (Fig. 4 B). In contrast with Th17-related cytokines, surface expression of NK cell receptors such as NKG2D, NKG2A/C/E, Ly49A/D, and Ly49I/G was comparable in eczematous and normal mice (unpublished data).

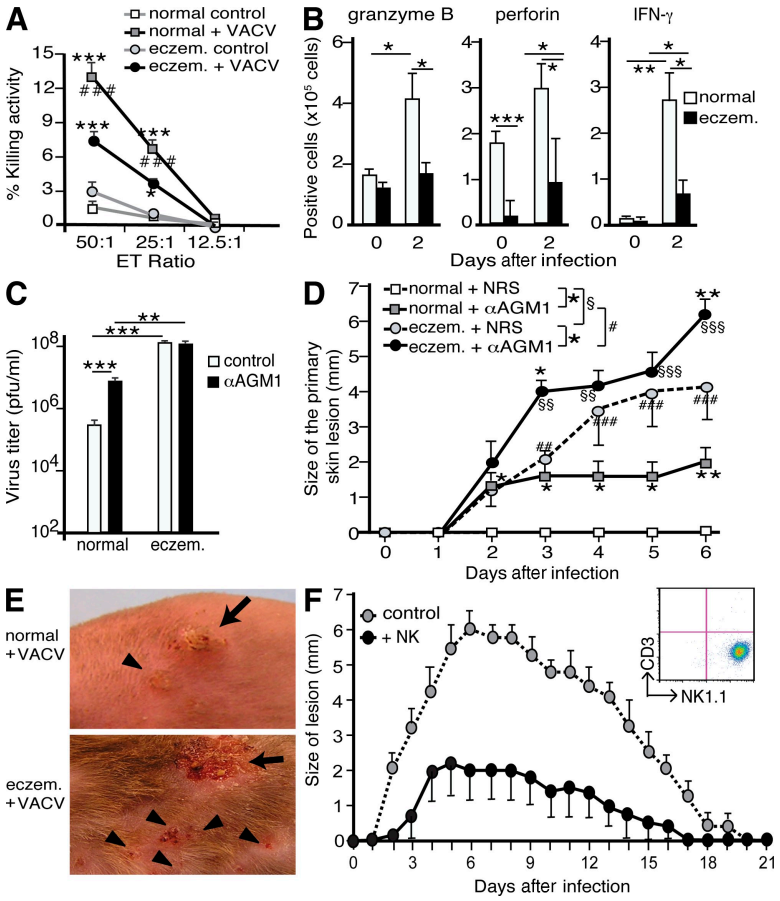

Figure 3. Reduced NK cell activity was critical for the development of VACV-induced erosive skin lesions in eczematous mice. (A) NK cell cytotoxic activity of splenocytes on day 2 after infection was measured using YAC-1 cells as target cells at the indicated effector-to-target ratios. Shown are results representative of 5 independent experiments. Data represent means and SEM values. *, $P < 0.05$; and ***, $P < 0.001$ versus uninfected mice. ###, $P < 0.001$ versus eczematous mice. (B) Flow cytometric analysis of $CD3^-$ $NK1.1^+$ NK cells expressing granzyme B, perforin, or IFN-γ in spleens. Shown are results representative of 8 independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. (C) Virus titers in spleens from αAGM1- or NRS-treated mice. (D) Sizes of primary skin lesions in αAGM1- or NRS-treated mice. *, comparison between αAGM1- and NRS-treated mice; #, comparison between αAGM1-treated normal and eczematous mice; and §, comparison between NRS-treated normal and eczematous mice (one, two, and three symbols indicate $P < 0.05$, 0.01, and 0.001, respectively. (E) Virus infection in αAGM1-treated normal mice induced erythematous papules at the inoculation site (indicated by the arrow; top) and satellite lesions. Satellite lesions were also induced in αAGM1-treated eczematous mice (indicated by arrowheads). Satellite lesions in both normal/αAGM1 and eczematous/αAGM1 mice were confirmed to contain live virus. Shown in C–E are representative results of three independent experiments (n = 4–8 mice per group). (F) Adoptive transfer of cultured NK cells. Sizes of primary skin lesions are shown. The inset shows the purity of transferred NK cells as analyzed by flow cytometry. Shown are results representative of three independent experiments. Data in A and C–F represent means and SEM values; data in B represent means and SD values.

Neutralization of IL-17A in eczematous mice with anti–IL-17 antibody caused a delay in the onset of skin lesions after virus infection, and the lesion size was significantly smaller on days 2 and 3 after infection (Fig. 4 C). Although the number and the percentage of NK cells in the spleen and at the lesion site were not changed by anti–IL-17 antibody treatment (Fig. 4 D), the proportions of NK cells expressing granzyme B, perforin, and IFN-γ were increased in IL-17–neutralized mice (Fig. 4 E). Consistent with these changes, viral loads in the spleen and lesional skin were lower in IL-17–neutralized mice (Fig. 4 F). Furthermore, when the NK cells were depleted by αAGM1 antibody, the effect of anti–IL-17 antibody treatment on the incidence and lesion size (Fig. 4 G) and viral titers (Fig. S4) was almost abrogated, indicating that effects of IL-17 neutralization are exerted through the regulation of NK cells. Consistent with these in vivo findings, the expression of killing

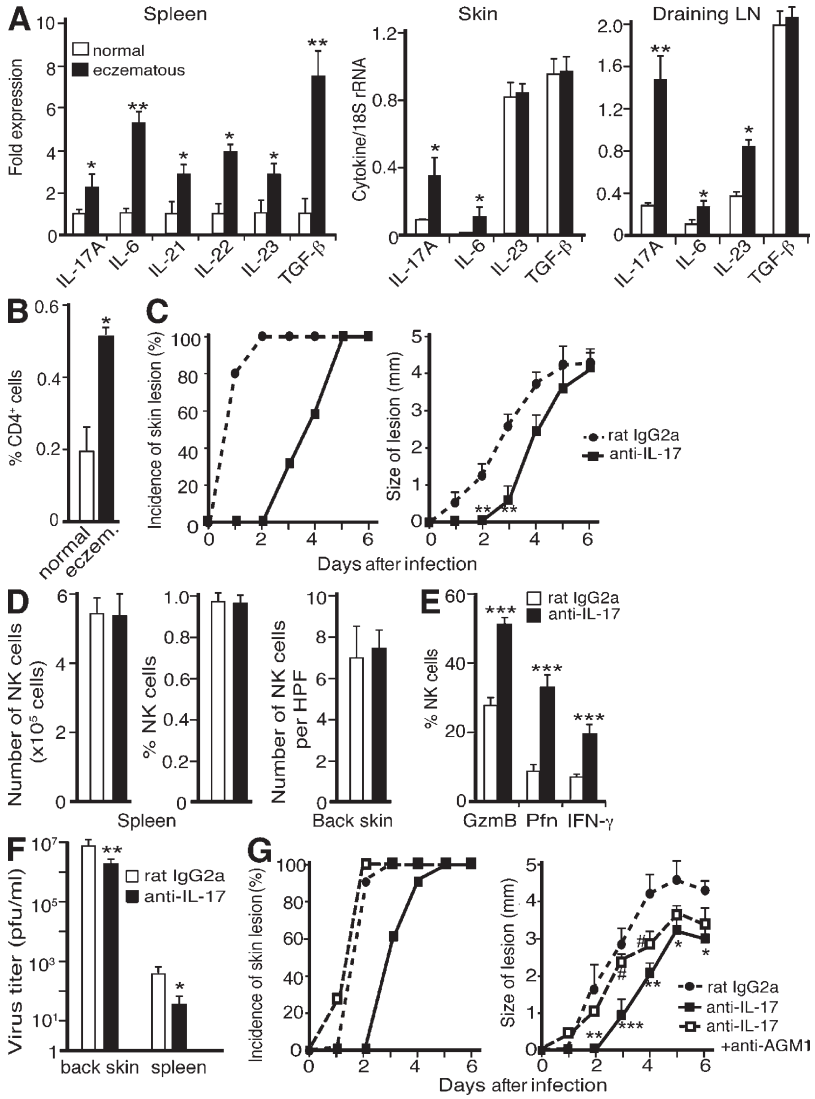

Figure 4. Role of IL-17A in reduced NK cell cytotoxicity in eczematous mice. (A) mRNA expression of IL-17A and Th17-related cytokines was analyzed by real-time PCR (spleen) or semiquantitative RT-PCR analysis (skin and draining lymph node). Values were normalized against those of normal mice. Shown are results representative of two independent experiments (n = 4–6 mice). *, $P < 0.05$; and **, $P < 0.01$ by the Student's t test. (B) $CD3^+CD4^+IL\text{-}17^+$ Th17 cells were enumerated in draining lymph nodes. (C) The onset of skin lesion development was delayed (left) and the size of primary skin lesions was smaller (right) in mice treated with anti–IL-17 mAb. **, $P < 0.01$ versus control. (D) NK cells in spleens and lesional skin were enumerated by flow cytometry and immunohistochemistry, respectively. (E) Splenic NK cells expressing granzyme B (GzmB), perforin (Pfn), or IFN-γ were analyzed by flow cytometry in mice treated with anti–IL-17 or control mAb day 2 after infection. (F) Virus titers were measured on day 7. Shown are representative results from three independent experiments. *, $P < 0.05$; and **, $P < 0.01$ versus control. (G) Mice were NK-depleted by αAGM1 injection 1 d before VACV infection. Anti–IL-17 antibody was also intraperitoneally injected 2 h after αAGM1 injection. After VACV infection, anti–IL-17 was injected on days 1 and 3, and αAGM1 was injected on days 2 and 5. Skin lesion development was observed and lesion size was measured for 6 d. The result is a representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus rat IgG2a–injected mice. #, $P < 0.05$ versus anti–IL-17 treated, NK-nondepleted mice. Data represent means and SEM values.

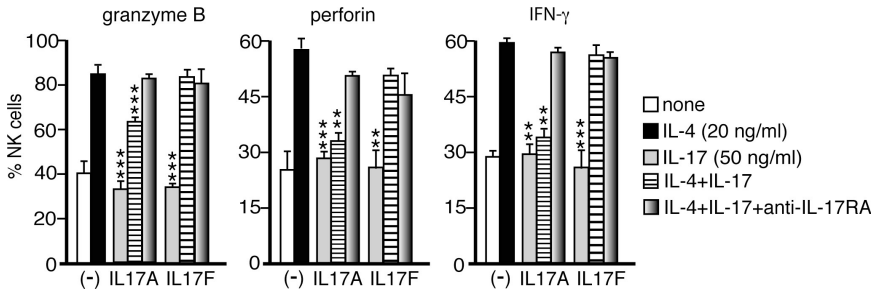

Figure 5. IL-17A but not IL-17F reduces NK cell cytotoxicity in vitro. Splenic NK cells were incubated with the indicated cytokines for 48 h before flow cytometric analysis of NK cells expressing granzyme B, perforin, or IFN-γ. Shown are results representative of two independent experiments. Data represent means and SEM values. **, $P < 0.01$; and ***, $P < 0.001$ versus IL-4 by one-way analysis of variance.

effector molecules in cultured splenic NK cells was reduced by IL-17A in a dose-dependent manner (Fig. S5), but not by IL-17F (Fig. 5). IL-17A reduced the expression of killing effectors induced by IL-4 (Fig. 5), IL-2, IL-12, IL-15, or IL-18 (Fig. S6). The survival of these NK cells was not affected by IL-17A or IL-17F (unpublished data). These results collectively suggest that IL-17A plays a critical role in lowering NK cell activity in eczematous mice.

IL-15 is required for the proliferation and activation of NK cells (16). Antibody-mediated neutralization of IL-15 caused more severe skin lesions in VACV-infected normal mice compared with the control cohort (Fig. S7). However, IL-15 neutralization in eczematous mice did not induce significant differences in skin lesion development. Although the mRNA level of IL-15 is not significantly different between normal and eczematous mice (unpublished data), the results of IL-15 neutralization further confirm that NK cell activity is critical for early protection from skin lesion development.

Our NC/Nga infection model does not exhibit all of the expected features of human eczema vaccinatum. For instance, NC/Nga mice with eczematous skin lesions exhibited functional but not numerical defects in NK cells, unlike atopic dermatitis patients, who have defects in both number and function (17, 18). Nevertheless, this model exhibits key features of atopic dermatitis observed in humans, including defective NK cell killing activity (17, 18) and high IL-17A expression (5, 6). IL-6 and TGF-β are required for induction of Th17 cells, and IL-23 is required for the establishment of Th17 cells (19, 20). IL-21 is produced by Th17 cells and exerts critical functions in Th17 cell differentiation (21–23). Th17 cells were more abundant and the Th17-related cytokines were increased in eczematous mice, suggesting that Th17 cells may be involved in reducing NK cell killing activity. The NK cell–suppressive function of IL-17A observed in our in vitro and in vivo studies was consistent with an earlier IL-17 study (24), although it is possible that the increased IL-17A and Th17-related cytokines might also contribute to VACV-induced inflammation via the enhanced immunopathology. Our results also support the conclusion that NK cells are important in controlling early local and systemic spreading of VACV in mice (25, 26). Although atopic dermatitis is still only partially understood in humans, there are strong indications that NK cell defects are involved (17, 18, 27). Our data now show that critical failures in NK cell–mediated immunity allow for disastrous early spread of vaccinia after cutaneous infection, and these NK cell defects are related to the immunosuppressive effects of IL-17A.

MATRIALS AND METHODS

Mouse infection. NC/Nga mice (10) were used in all animal experiments. Eczematous skin lesions were induced as described previously (11). In brief, mice were shaved on the back and dermatitis was induced by two rounds of a week-long treatment with *Dermatophagoides farinae* extract (Der f; Greer Laboratories) and SEB (Sigma-Aldrich). During this treatment, their back was occluded with a bandage, which was removed the following week. Mice with a clinical score of ≥8 (eczematous group) were intradermally injected with 10^6 PFU per 3 μl of VACV (Western Reserve strain) at the center of skin lesions. A cohort (normal group) of age and sex-matched mice with healthy skin was also infected at the same anatomical site. Clinical scores of eczematous skin lesions are based on severity (0, no signs; 1, mild; 2, intermediate; 3, severe) of four signs (redness, bleeding, eruption, and scaling). Scoring was performed in a blind manner. The virus was prepared by repeated (three times) freeze–thaw cycles of infected HeLa cells in DMEM/1% FCS followed by centrifugation. Uninfected HeLa cell extract was used as control. Virus titers were measured by plaque-forming assays on Vero cells. All of the mouse experiments were approved by an Institutional Review Board of the La Jolla Institute for Allergy and Immunology.

Histology. $CD4^+$ and $CD8^+$ T cells, Mac-1^+ monocytes/macrophages, and Ly49G2 (4D11)$^+$ NK cells were detected by immunochemical staining. Mast cells were stained by toluidine blue, and eosinophils and neutrophils were detected by hematoxylin and eosin or Congo red staining.

NK cell assay. NK cell activity was measured using YAC-1 cells as target cells, with effector-to-target ratios (12.5:1, 25:1, and 50:1) in spleen cells isolated on day 2 or 3 after infection.

NK cell cultures. Splenocytes from NC/Nga mice were negatively selected by MACS beads (Miltenyi Biotec) or the EasySep mouse NK cell enrichment kit (StemCell Technologies Inc.). These NK-enriched cells were cultured in RPMI 1640 with 10% FBS containing 500 ng/ml IL-15 for 4 d. The purity of NK cells was checked by flow cytometry after staining with anti-NK1.1 and anti-CD3 antibodies before the use for adoptive transfer. For in vitro NK cell assays, purified splenic NK cells or whole splenocytes were cultured in RPMI 1640 with 10% FBS containing 20 ng/ml IL-4 and/or 50 ng/ml IL-17A or IL-17F with or without 2 μg/ml anti–IL-17RA for 2 d, followed by flow cytometry.

Flow cytometry. Single-cell suspensions of splenocytes or lymph node cells were surface stained with anti-NK1.1 and CD3. The cells were then

fixed, permeabilized, and stained with anti–granzyme A, –granzyme B, -perforin, or –IFN-γ. Data were acquired with a FACSCalibur (BD) and analyzed using FlowJo software (Tree Star, Inc.).

RT-PCR. Skin tissues were taken from infection sites or erosive areas by punch biopsy, and axillary lymph nodes and spleens were also isolated. Total RNAs were isolated with TRIzol reagent (Invitrogen) and used as a template to prepare cDNAs. PCR products were analyzed by agarose gel elctrophoresis. Quantitative PCR was performed using a LightCycler 480 (Roche).

Statistical analysis. Statistical analysis in each independent experiment was performed with an unpaired, two-way analysis of variance using Prism software (GraphPad Software, Inc.), otherwise noted. $P < 0.05$ was considered statistically significant.

Online supplemental material. Fig. S1 shows antibody responses. Fig. S2 depicts αAGM1 effects on NK cells. Fig. S3 shows anti-NK1.1 effects. Fig. S4 depicts viral titers in anti–IL-17– and αAGM1-treated mice. Fig. S5 shows in vitro effects of IL-17A on NK cell mediators. Fig. S6 depicts the effects of various cytokines on NK cell mediators. Fig. S7 shows skin lesions in anti–IL-15–treated mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082835/DC1.

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