

Vaccinia virus inoculation in sites of allergic skin inflammation elicits a vigorous cutaneous IL-17 response

Michiko K. Oyoshi^{a,1}, Abdallah Elkhail^{a,1}, Lalit Kumar^{a,1}, Jordan E. Scott^a, Suresh Koduru^a, Rui He^a, Donald Y. M. Leung^b, Michael D. Howell^b, Hans C. Oettgen^a, George F. Murphy^c, and Raif S. Geha^{a,2}

^aDivision of Immunology, Children's Hospital and ^cDivision of Dermatopathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115; and ^bDepartment of Pediatrics, National Jewish Medical and Research Center, Denver, CO 80206

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Eczema vaccinatum (EV) is a complication of smallpox vaccination occurring in patients with atopic dermatitis. In affected individuals, vaccinia virus (VV) spreads through the skin, resulting in large primary lesions and satellite lesions, and infects internal organs. BALB/c mice inoculated with VV at sites of Th2-biased allergic skin inflammation elicited by epicutaneous ovalbumin (OVA) sensitization exhibited larger primary lesions that were erosive, more satellite lesions, and higher viral loads in skin and internal organs than mice inoculated in saline-exposed skin, unsensitized skin, or skin sites with Th1-dominant inflammation. VV inoculation in OVA-sensitized skin induced marked local expression of IL-17 transcripts and massive neutrophil infiltration compared to VV inoculation in saline-exposed skin. Treatment with anti-IL-17 decreased the size of primary lesions, numbers of satellite lesions, and viral loads. Addition of IL-17 promoted VV replication in skin explants. These results suggest that IL-17 may be a potential therapeutic target in EV.

atopic dermatitis | eczema vaccinatum

Vaccinia virus (VV) belongs to the orthopoxvirus genus of the Poxviridae family, which includes variola virus, the agent that causes smallpox, and is a potent vaccine against smallpox (1). Smallpox was eradicated in 1980 and vaccination ceased in the U.S. in 1972 and in the rest of the world in 1979 (1). Recent concern that VV might be used as a bioterror weapon has led to selective vaccination of military personnel and to contingency plans for mass vaccination. A major concern is that patients with atopic dermatitis (AD), which affects 15–20% of children in industrialized countries, are susceptible to eczema vaccinatum (EV), a life-threatening complication in which VV disseminates in the skin, resulting in satellite skin lesions, and spreads to internal organs (2). Although smallpox vaccination is contraindicated in AD patients, they are at risk for EV following contact with recently immunized individuals (3).

IL-4 and IL-10 promote VV spread (4, 5). In contrast, IFN- γ (6), IgG antibody (7), CD8⁺ cytotoxic T cells (8), NK cells (9), and the cathelicidin family of antimicrobial peptides (AMPs) (10) are thought to contribute to VV containment. It has been suggested that IL-4 driven down-regulation of AMPs in AD skin lesions predisposes to EV (10). IL-17 is expressed in acute skin lesions of patients with AD and in their peripheral blood CD4⁺ T cells (11, 12). There is controversy as to whether IL-17 promotes or attenuates VV infection (13, 14).

We have previously shown that epicutaneous (EC) sensitization of BALB/c mice with ovalbumin (OVA) results in skin inflammation with similarities to AD skin lesions, characterized by infiltration with CD4⁺ cells and eosinophils and by increased expression of mRNA for Th2 cytokines and IL-17, but not IFN- γ (15, 16). In this study, we show that inoculation of VV in OVA-sensitized skin results in larger primary lesions, more satellite lesions, and higher viral loads and markedly higher local expression of IL-17 than VV inoculation in saline-exposed skin or skin with Th1-dominant inflammation. We show that IL-17 promotes viral dissemination in this model.

Results

VV Inoculation at Sites of Allergic Skin Inflammation Results in Large Erosive Primary Lesions, Satellite Skin Lesions, and Increased Viral Spread. Eight days after infection, BALB/c mice inoculated with VV in OVA-sensitized skin developed significantly larger primary lesions that were erosive and pustular, as well as significantly higher numbers of satellite lesions than controls inoculated with VV in saline-exposed or unsensitized skin (Fig. 1*A–C*). VV genomes were significantly higher in the inoculated skin, ovaries, and kidneys of OVA-sensitized mice than in controls (Fig. 1*D* and *E*). When VV was inoculated distantly (>3 cm away) from the EC sensitization site, there was no difference between OVA-sensitized mice and controls (data not shown), indicating that EC sensitization with OVA did not cause a general impairment in the ability to contain VV.

OVA-sensitized skin of C57BL/6 mice exhibits less marked eosinophil infiltration than OVA-sensitized skin of BALB/c mice and up-regulates expression of IFN- γ , in addition to Th2 cytokines (17). C57BL/6 mice inoculated with VV in OVA-sensitized skin developed significantly larger primary lesions and higher viral loads in skin, ovaries, and kidneys than controls (Fig. S1). However, primary lesions were smaller than in BALB/c mice, and no satellite skin lesions were observed, possibly because of the local IFN- γ response of C57BL/6 mice. These results suggest that increased susceptibility to VV in mice inoculated at sites of allergic skin inflammation is not strain-specific.

BALB/c mice sensitized on abdominal skin with the hapten dinitrofluorobenzene (DNFB) and challenged 5 days later on back skin with hapten develop Th1-dominated skin inflammation with a striking increase in IFN- γ , but not IL-4 or IL-13 mRNA expression (18). BALB/c mice inoculated with VV at DNFB-challenged skin sites developed significantly smaller primary lesions than controls (Fig. 2*A*), with no detectable viral genomes. Furthermore, they did not exhibit satellite lesions, and viral loads in internal organs were comparable to controls (data not shown). These results suggest that VV inoculation into sites of Th2-dominated skin inflammation is selectively associated with increased susceptibility to the virus.

VV Inoculation in OVA-Sensitized Skin Elicits a Mixed, Modestly Th2 Skewed, Th Response. Splenocytes from mice inoculated with VV via OVA-sensitized skin secreted $\approx$ 5-fold more IL-4 and $\approx$ 2-fold more IFN- γ and IL-17 in response to VV than splenocytes from

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¹M.K.O., A.E., and L.K. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: raif.geha@childrens.harvard.edu.

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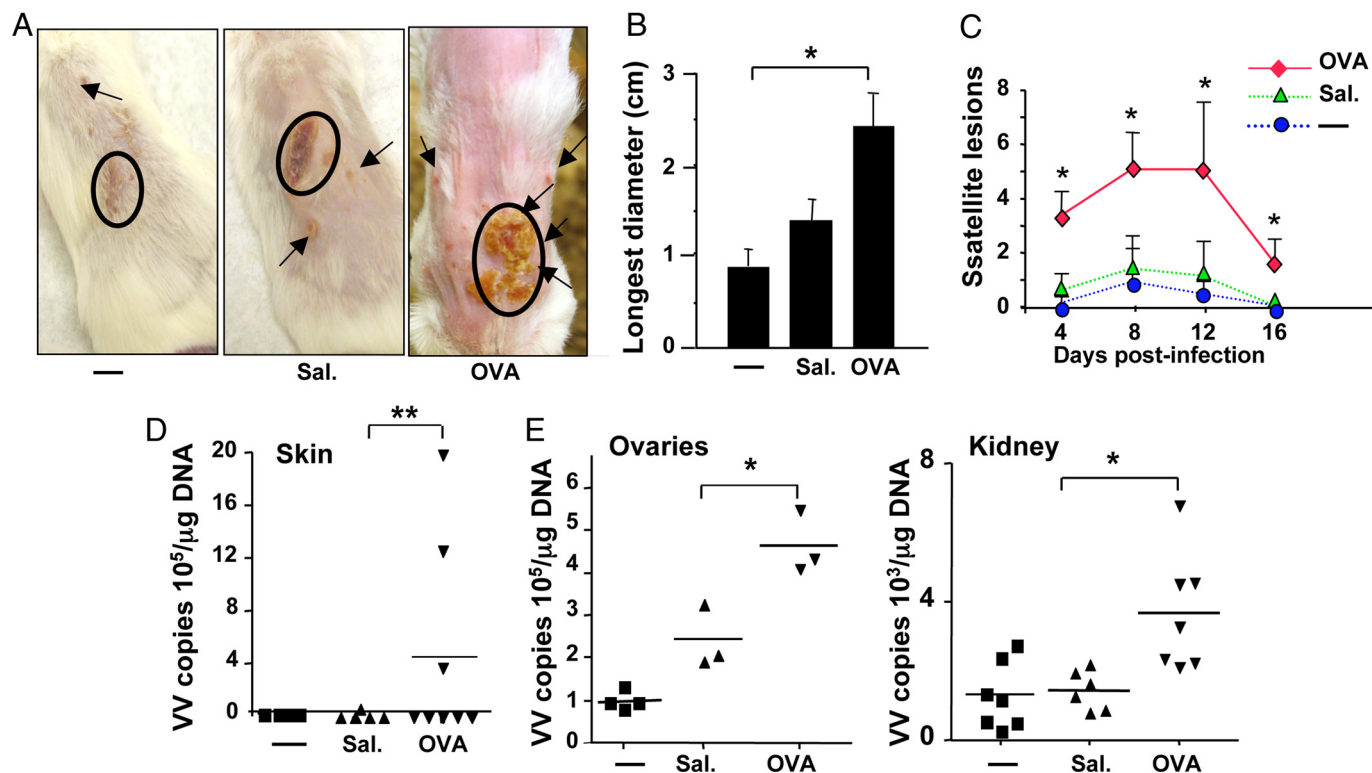


Fig. 1. Response of BALB/c mice to VV inoculation at sites of OVA sensitization. (A) Primary and satellite lesions in BALB/c mice inoculated with VV in unsensitized (–), saline-exposed (Sal.), and OVA-sensitized skin. (B) Largest diameter of primary lesion ($n = 4$ –5 per group). (C) Number of satellite lesions ($n = 5$ –12 per group). (D) Viral load in inoculated skin. VV genomes were detected in OVA-sensitized skin from seven of eight mice, but not in skin from five saline-exposed mice. (E) Viral load in internal organs ($n = 3$ –7 per group). Values and error bars represent mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$.

control mice (Fig. 3A). This resulted in a modest Th2 skewing with a ≈ 2.5 -fold decrease in the IFN- γ :IL-4 ratio. Accordingly, VV-specific serum IgG1 levels were significantly higher in mice inoculated with VV in OVA-sensitized skin than in control mice (Fig. 3B). VV-specific IgG2a levels were comparable (data not shown).

CD8⁺ cells are important in containing VV (8). H-2K^b/VV peptide pentamers for use in BALB/c mice are not available, but H-2K^b/VV TSYKFESV pentamers for use in C57BL/6 mice are. The frequencies of CD8⁺pentamer⁺ and of pentamer⁺IFN- γ ⁺ cells were comparable in spleens of C57BL/6 mice inoculated with VV via OVA-sensitized skin and controls (Fig. S2A and B). The numbers of splenic DX5⁺ NK cells and the level of NK cell cytotoxic activity against YAC-1 target cells were also comparable in the two groups (Fig. S2C and data not shown).

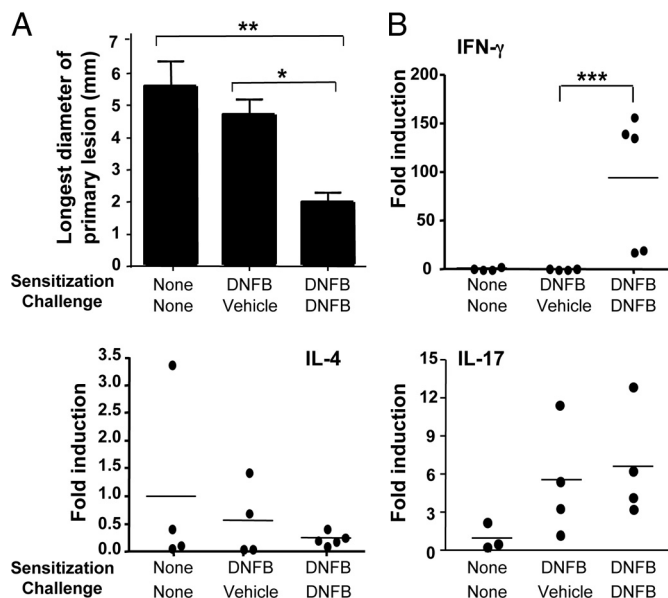
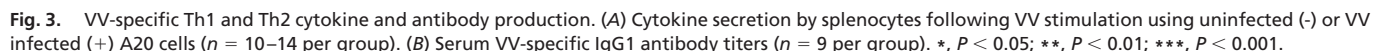


Fig. 2. Response of BALB/c mice to VV inoculation at sites of DNFB hapten challenge. (A) Longest diameter of primary lesion. (B) Cytokine mRNA expression ($n = 4$ –5 per group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

Selectively Increased IL-17 Expression in Sites of Allergic Skin Inflammation Inoculated with VV. We previously showed that IL-4 and IL-17 expression is up-regulated and CRAMP expression is down-regulated in OVA-sensitized skin of BALB/c mice (16, 19). Eight days after cessation of EC sensitization, expression of IL-4, IL-17, and CRAMP in OVA-sensitized skin was comparable to that in control skin sites (Fig. 4A), suggesting that the changes in expression of these cytokines in OVA-sensitized skin are dependent on persistent antigen exposure. Following VV inoculation, expression of IL-4 and IL-10, which promote VV spread, and of IFN- γ and CRAMP, which inhibit it, was comparable in VV-inoculated OVA-sensitized, saline-exposed, and unsensitized skin (Fig. 4A and (Fig. S3). In contrast, VV inoculation in OVA-sensitized EC-sensitized skin strongly up-regulated IL-17A expression to significantly higher levels than VV inoculation in saline-exposed or unsensitized skin. VV inoculation in OVA-sensitized skin also strongly and significantly up-regulated mRNA expression of the Th17 promoting cytokines IL-6 and IL-23 compared to inoculation in saline-exposed or unsensitized skin (Fig. 4B). IL-17A causes neutrophil accumulation by inducing the expression of neutrophil attracting CXC chemokines (20, 21). VV-inoculated unsensitized and saline-exposed control sites showed cutaneous and s.c. infiltration with



VV inoculation of DNFB-challenged skin of DNFB-sensitized mice caused strong up-regulation (≈ 90 -fold) of IFN- γ , but not IL-4 or IL-13, mRNA expression compared to VV inoculation of vehicle-challenged skin from these mice or of skin from unsensitized mice (Fig. 2*B* and data not shown). There was only modest (≈ 6 -fold) and comparable up-regulation of IL-17 expression in VV-inoculated hapten and vehicle-challenged skin sites of DNFB-sensitized mice relative to VV-inoculated skin of unsensitized mice (Fig. 2*B*). These results suggested that VV infection and Th2-dominated skin inflammation synergize to drive a vigorous IL17 response to the virus in inoculated skin.

IL-17 Promotes Viral Recovery in VV-Inoculated Skin Explants. We tested the possibility that local expression of IL-17 promotes VV replication in skin. Very few viral genomes could be detected 2 days after in vitro inoculation of unsensitized mouse skin explants from BALB/c mice. Addition of IL-17 resulted in significantly increased recovery of viral genomes (Fig. 64). IL-17 caused a significant

Addition of IL-17 had no significant effect on VV replication in the mouse keratinocyte cell line PAM212 or in mouse fibroblasts (data not shown), but significantly increased viral replication in mouse bone marrow-derived DCs (BMDCs) (Fig. 6D). This was specifically inhibited by anti-IL-17 mAb, but not control IgG.

Inoculation of VV in OVA-sensitized skin of BALB/c mice elicited a mixed systemic Th response evidenced by secretion of IL-4, IFN- γ , and IL-17 by splenocytes stimulated with VV-infected APCs. Splenocytes from mice inoculated in OVA-sensitized skin secreted higher amounts of all three cytokines than splenocytes

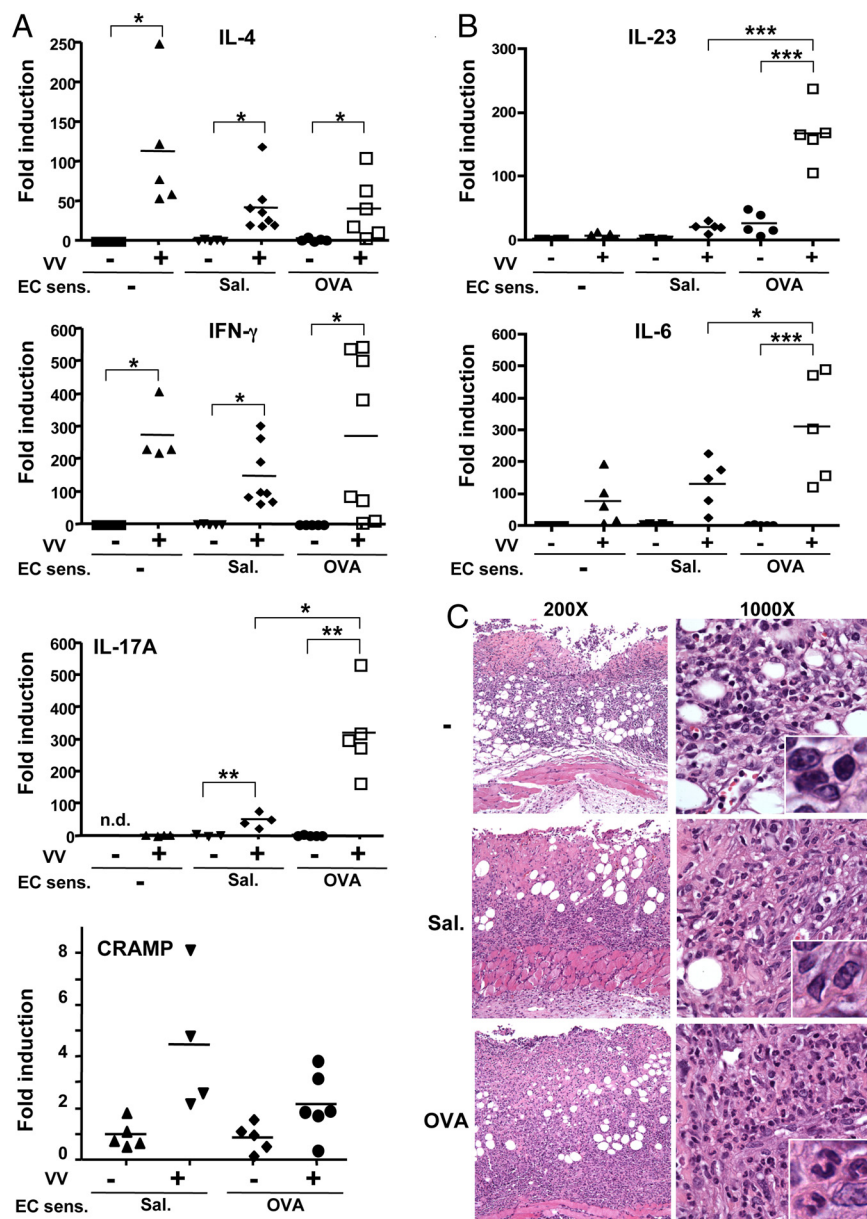


Fig. 4. Cytokine expression and histology of VV-infected skin. (A) Cytokine expression as fold induction relative to uninfected unsensitized skin. IL-17A mRNA levels were normalized to VV-infected unsensitized skin, as IL-17 mRNA was not detectable (n.d.) in uninfected unsensitized skin ($n = 4-8$ per group). (B) Expression of IL-23 and IL-6 ($n = 5$ per group). (C) Representative photomicrographs of vaccinia inoculation sites at 200 $\times$ and 1,000 $\times$; inset, 5 $\times$ photo enlargement of 1,000 $\times$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

from controls inoculated in saline-exposed or unsensitized skin. However, while the increases in IFN- γ and IL-17 secretion were comparable (≈ 2 -fold each), there was a relatively greater increase in IL-4 secretion (≈ 5 -fold), indicating modest Th2 skewing and consistent with the higher serum IgG1 anti-VV levels in these mice. We previously observed Th2 skewing in mice inoculated with the TK-WR strain in OVA-sensitized skin (19). The increased cytokine production across the board in mice inoculated in OVA-sensitized skin could be due to stronger antigenic stimulation resulting from increased antigenic load in the skin and possibly to increased VV access through inflamed skin. The relatively greater increase in IL-4 production might be explained by the observation that DCs from DLN of OVA-sensitized, but not saline-exposed, skin drive IL-4 production in naive T cells (data not shown).

Expression of IL-4 and IL-10, which promote VV spread, and IFN- γ and CRAMP, which inhibit it, was comparable in VV-

inoculated OVA-sensitized, saline-exposed, and unsensitized skin. In contrast, there was a striking local up-regulation of IL-17 mRNA expression in response to VV inoculation at sites of allergic inflammation, but not at sites of Th1-dominated inflammation. Strong expression of IL-17 in VV-infected OVA-sensitized skin was associated with a predominantly neutrophilic infiltrate and a purulent exudate, consistent with the known ability of IL-17 to induce neutrophil chemoattractants (20, 21), and with the tissue destructive character of Th17-dominated inflammation (24). There was markedly less IL-17 expression in VV-inoculated saline-exposed skin and very little in VV-inoculated unsensitized skin, and lymphocytes were the predominant infiltrating cells in these sites. The higher IL-17 mRNA expression in the skin of saline-sensitized mice compared to the skin of unsensitized mice, both before and after VV inoculation, may explain the bigger size of the primary lesions and the greater number of satellite lesions observed in the former.

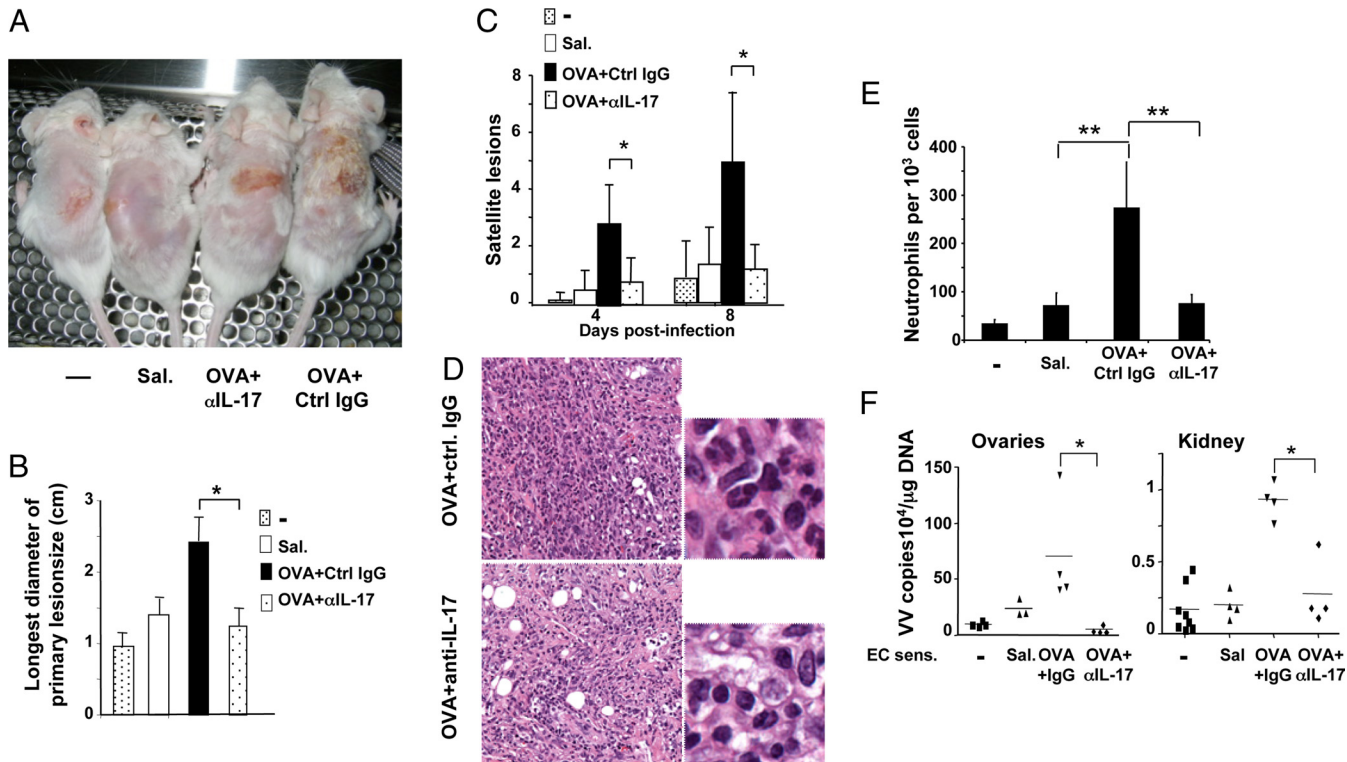


Fig. 5. Effect of anti-IL-17 treatment of mice inoculated with VV in OVA-sensitized skin. (A) Primary lesions 8 days postinfection. (B) Size of the largest diameter of the primary lesion ($n = 5-8$ mice per group). (C) Number of satellite lesions ($n = 5-8$ mice per group). (D) Skin histology (400 $\times$, H&E stain). (E) Numbers of neutrophils in skin per 1,000 cells counted ($n = 4$ mice per group). (F) Viral load in internal organs. *, $P < 0.05$; **, $P < 0.01$.

VV inoculation induced significantly higher levels of the Th17-promoting cytokines IL-6 and IL-23 in OVA-sensitized skin compared to saline-exposed or unsensitized skin. Increased local IL-6

and IL-23 expression would amplify IL-17 production by skin homing Th17 cells and explain the higher expression of IL-17 in VV-inoculated OVA-sensitized skin.

Treatment of BALB/c mice inoculated with VV via OVA-sensitized skin with anti-IL-17 reduced the size of the primary lesions, the number of satellite lesions, and the viral load in internal organs to levels comparable to those in control mice inoculated in saline-exposed skin. Treatment with anti-IL-17 caused no changes in Th1 or Th2 cytokine production by splenocytes, NK cell activity, IL-4, IFN- γ , or CRAMP expression in the skin that would favor VV containment. IL-17 directly enhanced VV replication in mouse skin explants, as evidenced by increased recovery of VV genomes and expression of the replication associated viral gene E3L. Furthermore, neutralization of IL-17 inhibited VV replication in explanted OVA-sensitized skin, which expresses IL-17 mRNA, probably derived from infiltrating T cells, as it contains very few, if any neutrophils (16). Recently IL-17 was shown to promote replication of Theiler's murine encephalomyelitis virus by enhancing the survival of virus-infected cells and blocking target cell destruction by cytotoxic T cells (25). IL-17 promoted VV replication in mouse BMDCs, but not fibroblasts or the keratinocyte cell line PAM212, suggesting that increased viral replication in skin DCs may contribute to the promoting effect of IL-17 on VV replication in skin explants. We cannot rule out additional direct effects of IL-17 on VV replication on other skin cells such as Langerhans cells. It is also possible that IL-17 might induce the production of cytokines/factors by DCs, or other skin cells, that promote viral replication in a paracrine manner.

Two previous studies explored the role of IL-17 in VV immunity and reached opposite conclusions. In these, mice were infected i.p. with VV expressing IL-17 (VV-IL-17). In one study, VV-IL-17 was more virulent than WT virus (13), while in the other it was less virulent (14). The reason for the discrepancy is not clear. Unlike VV

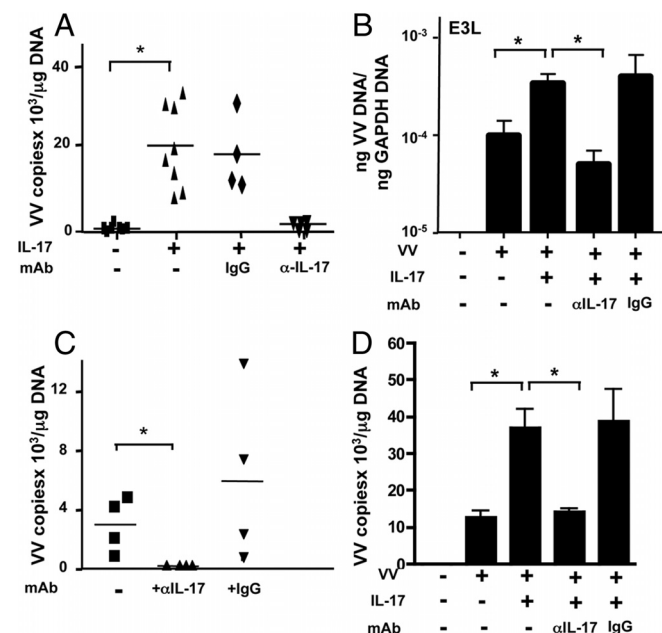


Fig. 6. IL-17 promotes VV recovery in skin explants. (A) VV genomes in VV inoculated skin explants. (B) Real-time RT-PCR analysis of E3L gene mRNA expression ($n = 5$). (C) Effect of anti-IL-17 mAb on the recovery of VV genomes from inoculated explants of OVA-sensitized skin. (D) VV genomes in BMDC culture. *, $P < 0.05$.

inoculation in the skin, which mimics human vaccination, i.p. injection of VV does not cause the development of skin lesions and leads to death at higher doses of VV. Moreover, in mice infected with VV-IL-17, the tissue distribution and level of expression of IL-17 are unlikely to reflect those elicited by vaccinia immunization.

In our study, the relative expression of IL-17 to the protective cytokine IFN- γ was markedly higher in OVA-sensitized skin inoculated with OVA compared to saline-exposed skin inoculated with VV. In contrast, the ratio of production of these two cytokines by splenocytes was comparable in mice inoculated with VV in OVA-sensitized skin and controls. This selective increase in cutaneous IL-17 expression may explain the observation that the major effects of VV inoculation at sites of allergic skin inflammation were observed at the site of inoculation while viral loads in internal organs were only modestly (≈ 2 –3-fold) increased. Since IL-17 is expressed in human AD skin lesions (11, 12), therapies that target IL-17 might be useful in limiting VV dissemination in patients with AD.

Materials and Methods

Mice. Mice were purchased from Jackson Laboratories and kept in a pathogen-free environment. All procedures performed on the mice were in accordance with the Animal Care and Use Committee of the Children's Hospital.

Vaccinia Virus. Vaccinia Virus Western Reserve strain (VR-1354) was obtained from American Type Culture Collection and expanded in monkey CV1 cells as previously described (19).

EC Sensitization, Hapten Challenge, and VV Inoculation. EC sensitization with OVA, sensitization with DNFB, and VV inoculation of 4- to 6-week-old female mice were performed as previously described (18, 19). Skin was examined 8 days after inoculation.

Quantitative PCR Analysis of VV Genomes. Tissue samples were immediately frozen and stored at -80°C . Quantification of VV genomes was performed as previously described (19). Viral genome measurements by PCR correlated with viral titers measurements by plaque assay.

In Vitro Cytokine Production and VV-Specific IgG2a and IgG1 Antibody ELISA.

These assays were performed as described previously (19), except that VR-1354 virus was used to infect A20 cells and coat ELISA plates.

Analysis of mRNA Expression for Cytokines and CRAMP in Skin. These analyses were performed as described before (16, 19).

Histological Analysis. Multiple 4- μm sections were stained with hematoxylin and eosin (H&E). The number of neutrophils among 1,000 cells was counted, blinded from four independent samples examined.

Anti-IL-17 Treatment. At days -1 , 0 , and $+2$ of VV inoculation, mice received intravenously 100 μg neutralizing IgG anti-mouse IL-17 mAb (clone 50104) or IgG control mAb (R&D Systems) in 100 μL .

VV Replication in Skin Explants. Skin explants were prepared, cultured, and inoculated with VV as previously described (10). IL-17 (250 ng/mL; R&D Systems) was added alone or together with 50 ng/mL anti-IL-17 mAb or IgG isotype control (R&D Systems). After 48 h, the explants were extensively washed. DNA was analyzed for viral genomes (19). RNA was isolated, and 1 μg RNA was reverse-transcribed, and real-time RT-PCR was performed for E3L and GAPDH as previously described (10).

DC Generation. DCs from BALB/c WT mice were generated as described (26). Briefly, bone marrow was isolated from the long bones of the hind legs of BALB/c WT mice. Cells (2×10^6) were plated in 100-mm dishes in complete RPMI medium 1640 (Invitrogen) supplemented with 20 ng/mL GM-CSF (PeproTech). On day 6, cells were cultured in the presence and absence of IL-17 and/or anti-IL-17 for 24 h before stimulation with VV. Cells were then infected with 0.1 pfu/cell VV for 24 h. After incubation, cells were washed with media to remove the remaining VV, then VV genomes were quantified.

Statistical Analysis. Significance of differences between the means was analyzed by using nonparametric Mann-Whitney U test.

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Supporting Information

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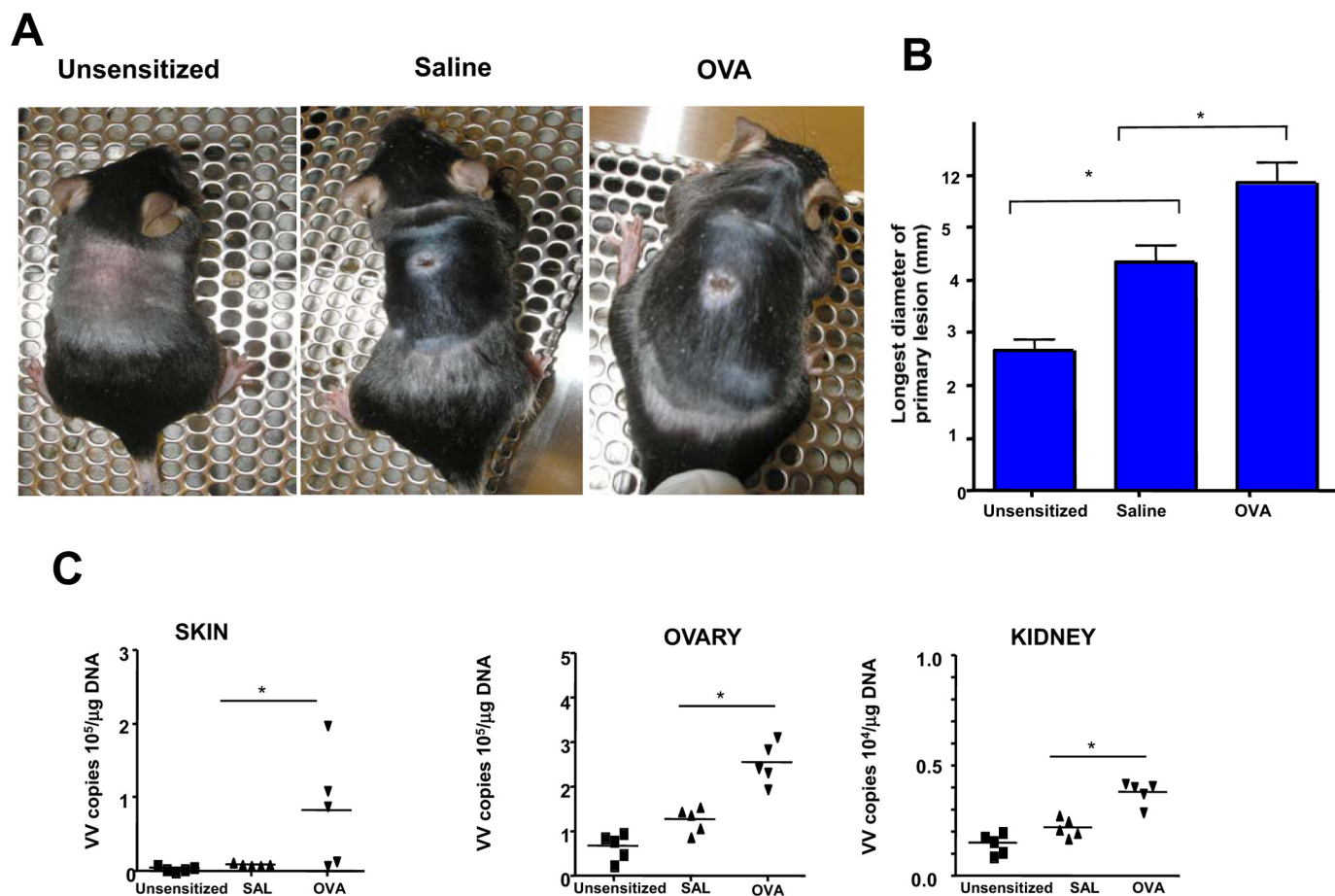


Fig. S1. Response of C57BL/6 mice to VV inoculation in EC sensitized skin. (A) Primary lesions in mice inoculated with VV in skin that was unsensitized, saline-exposed, or OVA-sensitized. (B) Largest diameter measurement of primary lesion. (C) Viral load in inoculated skin, ovary, and kidney. Values and error bars represent mean $\pm$ SD. $n = 5$ mice per group. *, $P < 0.05$.

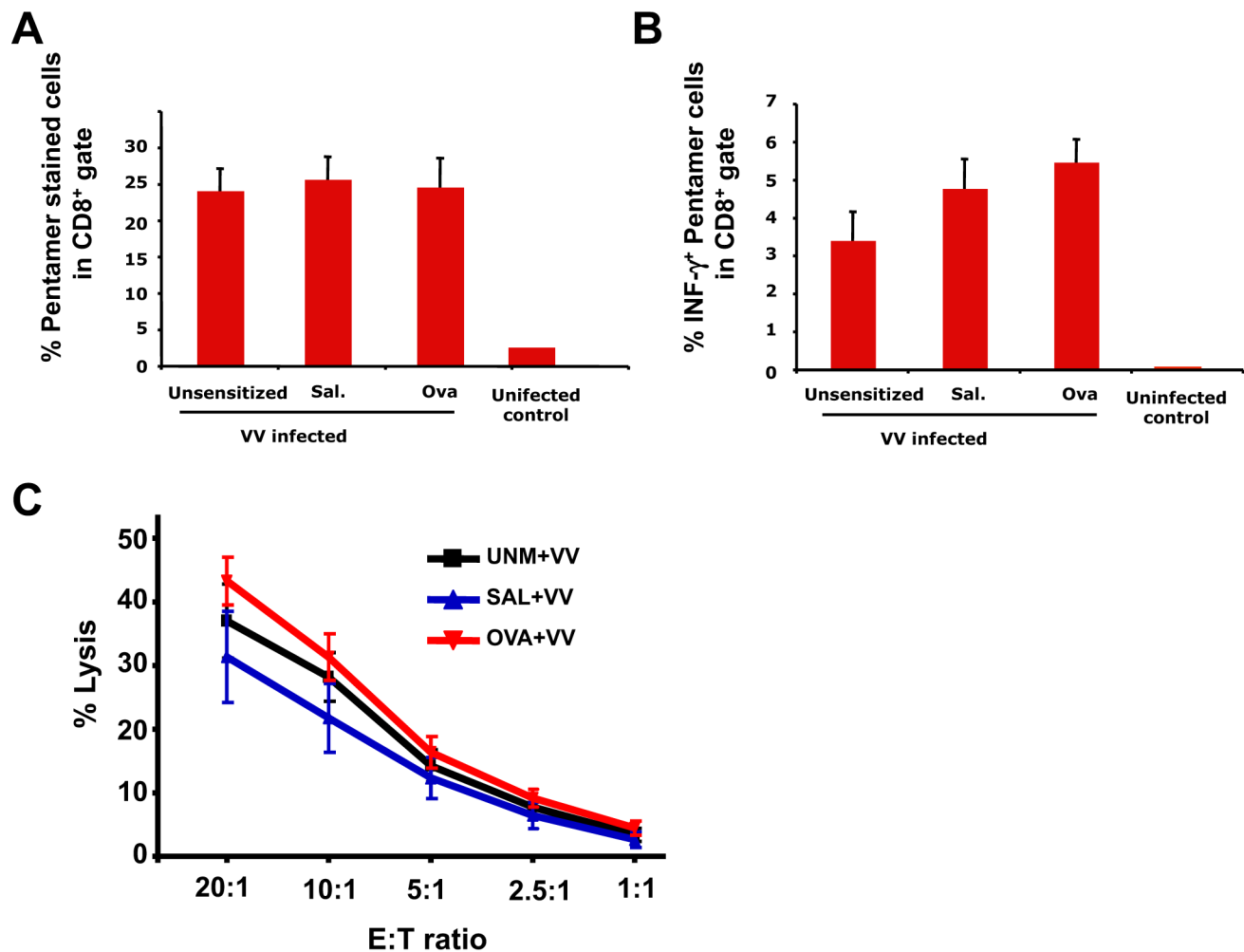


Fig. S2. VV-specific CD8⁺ cells, IFN-γ⁺ cells, and NK cell activity in spleens of mice inoculated with VV in EC-sensitized skin. (A and B) Percentage of CD8⁺ cells staining with MHC class I/VV-peptide pentamer (A) and of pentamer⁺IFN-γ⁺ cells (B) in C57BL/6 mice inoculated with VV in EC-sensitized skin. (C) Cytotoxic activity of NK cells purified by negative selection from spleens of BALB/c mice inoculated with VV in EC-sensitized skin and tested against YAC-1 target cells at various effector:target (E:T) ratios. *n* = 5–6 mice per group, for each experiment shown. **, *P* < 0.01 by ANOVA.

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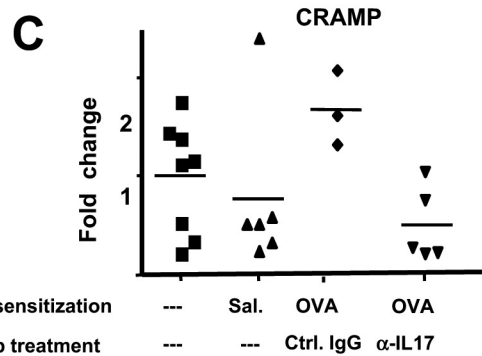
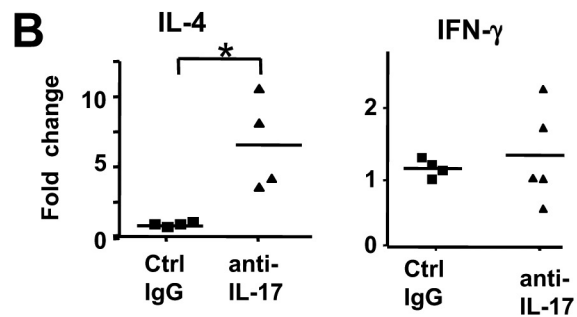
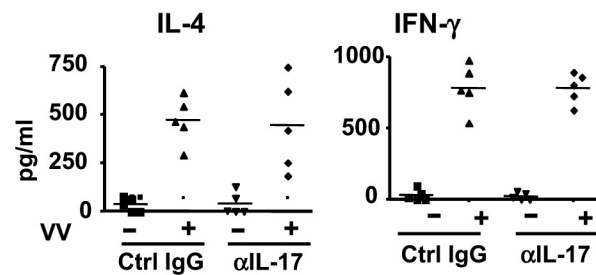


Fig. S4. Effect of anti-IL-17 treatment of mice VV inoculated in OVA-sensitized skin. (A) VV specific Th1 and Th2 cytokine production by splenocytes. (B and C) Th1 and Th2 cytokine expression (B) and CRAMP expression (C) in VV-inoculated skin. *, $P < 0.05$.