

Filaggrin deficiency promotes the dissemination of cutaneously inoculated vaccinia virus

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Background: Eczema vaccinatum is a life-threatening complication of smallpox vaccination in patients with atopic dermatitis (AD) characterized by dissemination of vaccinia virus (VV) in the skin and internal organs. Mutations in the filaggrin (*FLG*) gene, the most common genetic risk factor for AD, confer a greater risk for eczema herpeticum in patients with AD, suggesting that it impairs the response to cutaneous viral infections.

Objective: We sought to determine the effects of *FLG* deficiency on the response of mice to cutaneous VV inoculation.

Methods: VV was inoculated by means of scarification of unsensitized skin or skin topically sensitized with ovalbumin in *FLG*-deficient flaky tail (*ft/ft*) mice or wild-type (WT) control mice. The sizes of primary and satellite skin lesions were measured, and hematoxylin and eosin staining was performed. VV genome copy numbers and cytokine mRNA levels were measured by using quantitative PCR.

Results: VV inoculation in unsensitized skin of *ft/ft* mice, independent of the matted hair mutation, resulted in larger primary lesions, more abundant satellite lesions, heavier viral loads in internal organs, greater epidermal thickness, dermal cellular infiltration, and higher local *Il17a*, *Il4*, *Il13*, and *Ifng* mRNA levels than in WT control mice. VV inoculation at sites of topical ovalbumin application amplified all of these features in *ft/ft* mice but had no detectable effect in WT control mice. The number of satellite lesions and the viral loads in internal organs after cutaneous VV inoculation were significantly reduced in both unsensitized and topically sensitized *ft/ft;Il17a^{-/-}* mice.

Conclusion: *FLG* deficiency predisposes to eczema vaccinatum. This is mediated primarily through production of IL-17A. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *Vaccinia virus*, filaggrin, IL-17A

Abbreviations used

AD: Atopic dermatitis
EC: Epicutaneous
EH: Eczema herpeticum
EV: Eczema vaccinatum
FLG: Filaggrin
H&E: Hematoxylin and eosin
OVA: Ovalbumin
WT: Wild-type
VV: Vaccinia virus

Atopic dermatitis (AD) is a chronic pruritic inflammatory skin disorder that affects more than 15% of children in the United States.¹ AD is characterized by a defect in skin barrier function that results in dry itchy skin and is aggravated by skin injury inflicted by scratching.² Patients with AD are prone to T_H2-dominated immune responses, as well as bacterial and viral infections of the skin.³⁻⁵ In particular, they are susceptible to dissemination of viruses, such as vaccinia virus (VV) and herpes simplex virus,^{6,7} resulting in eczema vaccinatum (EV) and eczema herpeticum (EH), respectively. In patients with EV, VV spreads through the skin, resulting in large primary lesions surrounded by satellite lesions, and infects internal organs. Although smallpox was declared eradicated in 1979, recent fears that variola virus might be used as a biological weapon, along with concerns regarding the EV susceptibility of unimmunized atopic populations, have prompted the development of new safer vaccines. Predicting the efficacy of such vaccines in the absence of human smallpox depends on understanding the mechanisms of the disease. The reasons why patients with AD are at risk for EV are not known.

Strong genetic associations exist between mutations in the filaggrin (*FLG*) gene and AD.⁸⁻¹⁰ The *FLG* gene encodes for profilaggrin, the major component of keratohyalin granules. Patients with AD who carry *FLG* mutations have more persistent disease, a higher incidence of skin infections with herpes virus that result in EH,^{11,12} and a greater risk of multiple allergies, including asthma, allergic rhinitis, and peanut allergy.^{13,14} Whether *FLG* deficiency predisposes to EV has not been studied.

Flaky tail (*ft/ft*) mice carry a 5303delA frameshift mutation in the *FLG* gene, which is in linkage disequilibrium with a second mutation at the matted (*ma*) locus that confers hair matting.^{15,16} *ft/ft* mice have skin barrier dysfunction.¹⁷ We and others have previously reported that the homozygous *ft* mutation on an outbred genetic background is associated with spontaneous development of eczematous skin lesions; increased mRNA expression of *Il17a*, *Il4*, *Il13*, and *Ifng* in the skin; and increased serum IgE levels.^{17,18} After topical cutaneous exposure to ovalbumin (OVA), *ft/ft* mice on a mixed background have allergic skin inflammation evidenced by epidermal thickening, dermal

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Supported by National Institutes of Health/National Institute of Allergy and Infectious Diseases (NIAID) contract no. HHSN272201000020C (to R.S.G.). M.K.O. was supported by the Boston Children's Hospital Faculty Career Development Fellowship and the Children's Hospital Pediatric Associates Award.

Disclosure of potential conflict of interest: M. K. Oyoshi has received research support from the Boston Children's Hospital Faculty Career Development Fellowship/Children's Hospital Pediatric Associates Award. R. S. Geha has received research support from the Atopic Dermatitis Research Network (HHSN272201000020C). The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication July 11, 2014; revised December 11, 2014; accepted for publication December 12, 2014.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2014.12.1923>

CD4⁺ cell infiltration, and exaggerated local and systemic T_H17 and T_H2 responses.¹⁸ In contrast, topical application of OVA elicited no detectable local or systemic immune responses in wild-type (WT) BALB/c and C57BL/6 mice.¹⁸ In WT mice tape stripping, a surrogate for human scratching, is a prerequisite for induction of allergic skin inflammation and systemic immune responses to epicutaneous (EC) application of antigen. Genetic and environmental factors favoring induction of a cutaneous T_H2 response lead to impaired effector immune responses and ineffective elimination of VV and herpes simplex virus in mice, resulting in phenotypes analogous to human EV and EH.^{19–22} Animal studies support the hypothesis that skin barrier dysfunction caused by *FLG* mutations or mechanical injury secondary to scratching plays a key role in antigen sensitization, which leads to the development of AD.

To understand whether *FLG*-deficient mice are susceptible to development of EV, we backcrossed the mutant *FLG* allele of the *fl/fl* strain onto the T_H2-prone BALB/c background. This inbred strain has been well characterized with respect to models of allergic skin inflammation and EV.^{19,23–28} Transferring the *fl/fl* genotype into this background permits rigorous analyses of *fl/fl* phenotypes and a comparison of the responses of the mutant and WT BALB/c control mice. In this study we demonstrate that *fl/fl* mice inoculated with VV in the skin have more severe skin lesions, greater viral dissemination, and more intense cutaneous inflammation than WT control mice, indicating that *FLG* deficiency impairs VV containment. All these features were exaggerated in *fl/fl* mice inoculated with VV in skin topically sensitized with OVA, indicating that *FLG* deficiency and allergic inflammation synergize to impair VV containment. IL-17A was shown to mediate the susceptibility of *fl/fl* mice to VV because EV features were markedly attenuated in *fl/fl;il17a*^{−/−} mice.

METHODS

Mice

Flaky tail (*fl/fl*) mice from the Jackson Laboratory (Bar Harbor, Me) were backcrossed onto BALB/c mice from Charles River Laboratories (Wilmington, Mass). *fl/fl* mice on a BALB/c background were subsequently crossed with *il17a*^{−/−} BALB/c mice to generate *fl/fl;il17a*^{−/−} mice. Later in the course of the study, by extensive backcrossing, we were able to remove the matted hair mutation (*ma*) present in the original mixed strain. The mice with the removed *ma* mutation were designated *fl/fl.ma*^{wt/wt} mice. The sample size in each group was 5. All mice were bred in our animal facility, kept in a specific pathogen-free environment, and fed an OVA-free diet. All procedures performed on the mice were in accordance with the Animal Care and Use Committee of Boston Children's Hospital.

Topical skin sensitization and VV inoculation

The dorsal skin of anesthetized 6- to 8-week-old female mice was shaved and rested for 2 days for recovery from any injury by shaving, and then 100 μg of OVA (Grade V; Sigma, St Louis, Mo) in 100 μL of normal saline or placebo (100 μL of normal saline) was placed on a patch of sterile gauze (1 × 1 cm), which was secured to dorsal skin with a transparent bio-occlusive dressing (Tegaderm; Westnet, Canton, Mass). Each mouse had a total of three 1-week exposures to the patch separated by 2-week intervals.

Shaved dorsal skin of unsensitized mice or the sensitized site of topically sensitized mice was inoculated with VV Western Reserve strain (VR-1354; American Type Culture Collection, Manassas, Va) by means of skin scarification with 10⁷ plaque-forming units per mouse. Skin was examined 7 days after inoculation (see Fig E1 in this article's Online Repository at

www.jacionline.org). Lesion sizes were analyzed with the National Institutes of Health's Image software Image J (National Institutes of Health, Bethesda, Md). No mortality was observed after VV inoculation.

Histologic analysis

Multiple 4-μm sections of skin were stained with hematoxylin and eosin (H&E). Neutrophils were counted in a blind fashion in 10 to 15 high-power fields at a magnification of ×400. The epidermal thickness of 10 different randomly chosen sites was measured in each skin section from each mouse at a magnification of ×400.

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.²⁰ Cytokine mRNA expression was shown as fold induction relative to that seen in uninfected WT skin unless otherwise specified. Viral genome measurements obtained by using PCR correlated with viral titer measurements obtained by using a plaque assay.

Quantitative PCR analysis of cytokines

Total RNA was extracted from homogenized skin tissue or from cultured cells with the RNAqueous Extraction Kit (Ambion, Life Technologies, Grand Island, NY), according to the manufacturer's instructions. cDNA was generated with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, Calif). Quantitative real-time PCR was done with the TaqMan Gene Expression Assay, universal PCR master mix, and ABI Prism 7300 Sequence Detection System with commercial primers and probes, all from Applied Biosystems (Foster City, Calif). Fold induction of target gene expression was calculated by using the comparative method for relative quantitation by means of normalization to the internal control β₂-microglobulin, as described previously.²⁹

Measurement of *in vitro* cytokine production and VV-specific IgG_{2a} antibody ELISAs were performed, as described previously.³⁰

Statistical analysis

The Mann-Whitney *U* test was used to compare the distribution of each outcome between 2 groups. A Bonferroni-corrected significance level of .05 was used to account for pairwise comparisons among 3 or more groups. All analyses were performed with GraphPad Prism software, version 5.0 (GraphPad Software, La Jolla, Calif).

RESULTS

fl/fl mice are susceptible to cutaneous VV inoculation

We tested the hypothesis that *fl/fl* mice are predisposed to the development of EV. Seven days after VV inoculation in the back skin, *fl/fl* mice had significantly larger primary lesions and significantly higher numbers of satellite lesions than WT BALB/c control mice (Fig 1, A–C). Furthermore, they exhibited significantly higher viral loads in internal organs than WT control mice (Fig 1, D). These results suggest that *fl/fl* mice are more susceptible to having features of EV after inoculation with VV.

Inoculation of *fl/fl* mice with VV resulted in a significant increase in epidermal thickness, cellular infiltration of the dermis by mononuclear cells, and skin *Il4*, *Il13*, and *Ifng* mRNA levels compared with those seen in uninfected *fl/fl* mice or VV-inoculated WT control mice (Fig 1, E, F, and H). Inoculation of WT control mice with VV resulted in a slight but significant increase in epidermal thickness and skin *Il4*, *Il13*, and *Ifng* mRNA levels compared with those seen in uninfected WT control

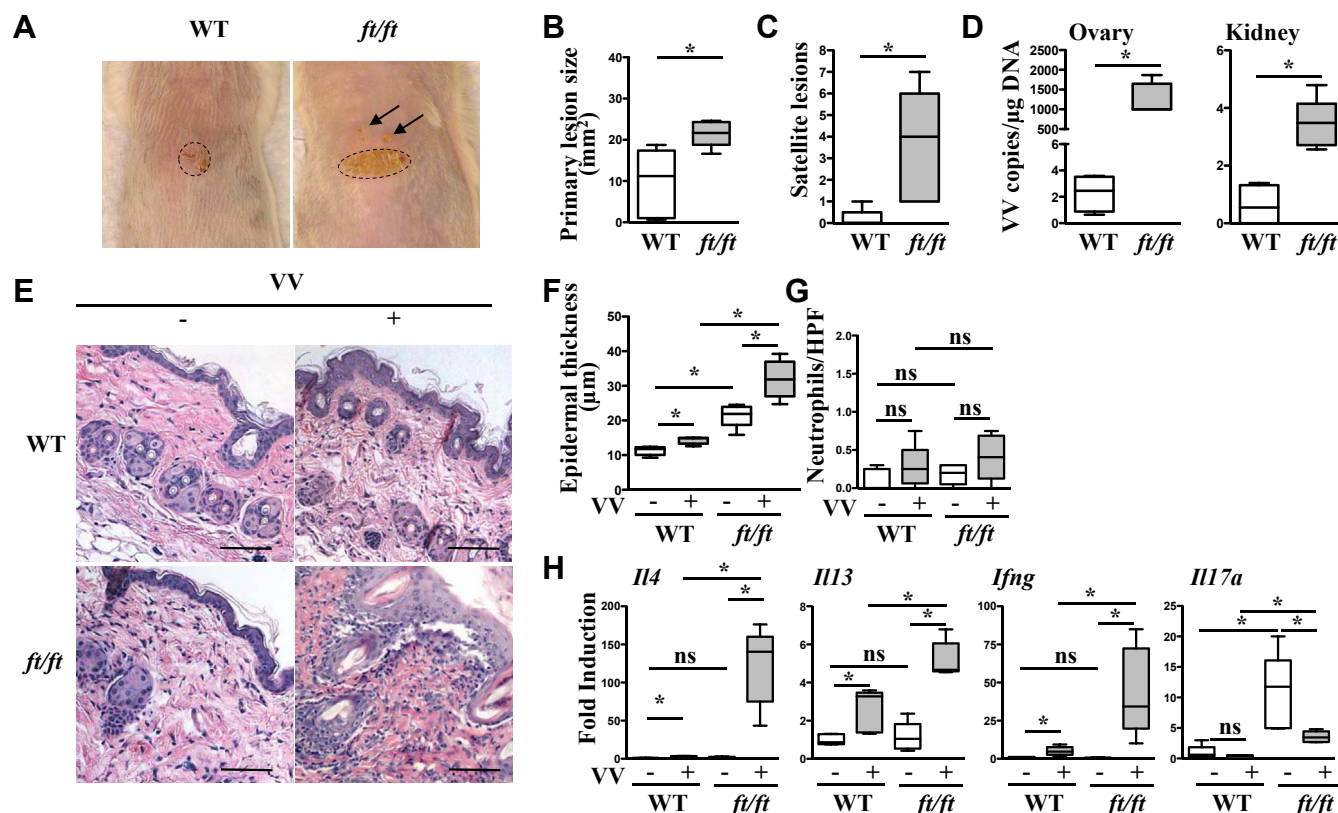


FIG 1. *ft/ft* mice have features of EV in response to cutaneous VV inoculation. **A–C,** Gross appearance (Fig 1, A), area of primary lesions (Fig 1, B), and number of satellite lesions (Fig 1, C). Dashed circles indicate primary lesions. Arrows indicate satellite lesions. **D,** Viral load. **E–H,** H&E-stained skin sections (×200 magnification; Fig 1, E), epidermal thickness (Fig 1, F), number of neutrophils (Fig 1, G), and cytokine mRNA expression (Fig 1, H). Scale bars = 100 μm. The line in the box indicates the median, and the whiskers represent the minimum to the maximum (n = 5 per group). **P* < .05. ns, Not significant.

mice (Fig 1, E, F, and H). However, no increase in skin-infiltrating neutrophil counts was observed in any of the experimental groups (Fig 1, E and G). Epidermal thickness and skin *Il17a* mRNA expression were increased in the skin of uninfected *ft/ft* mice compared with that seen in uninfected WT control mice (Fig 1, F and H), as previously reported in *ft/ft* mice on a mixed background.¹⁸ The high basal levels of skin *Il17a* mRNA in *ft/ft* mice were significantly decreased after VV inoculation but remained higher than the *Il17a* mRNA levels in the skin of VV-inoculated WT mice (Fig 1, H). VV inoculation had no detectable effect on *Il17a* mRNA in the skin of WT control mice. Splenocytes from *ft/ft* and WT control mice inoculated with VV secreted comparable amounts of IL-4, IL-13, IFN-γ, and IL-17A in response to *in vitro* stimulation with VV (see Fig E2, A, in this article's Online Repository at www.jacionline.org). Serum levels of VV-specific IgG_{2a} were higher in VV-inoculated *ft/ft* mice than in WT control mice (see Fig E2, B). These results demonstrate that *ft/ft* mice mount exaggerated cutaneous T_H2 and T_H1 responses to VV infection.

Recently, it has become evident that FLG-null (*Flg*^{-/-}) mice differ from *ft/ft* mice in that they do not experience spontaneous dermatitis and do not have abnormal transepidermal water loss.³¹ However, *Flg*^{-/-} mice, like *ft/ft* mice, mount a T_H2 response to topical sensitization of antigen.³¹ The differences between *ft/ft* mice and *Flg*^{-/-} mice raised the possibility that the homozygous *ma* mutation linked to the *ft* mutation might

contribute to the susceptibility of *ft/ft* mice to cutaneous inoculation with VV. To address this issue, we backcrossed our *ft/ft* mice on a BALB/c background to generate *ft/ft* mice that lack the *ma* mutation, as evidence by the lack of matted hairs and based on genomic sequencing for the *ma* mutation (data not shown).³² These mice were indistinguishable from *ft/ft* mice, which harbor the *ma* mutation in their susceptibility to VV infection, as measured by the size of the primary lesion, the number of satellite lesions, viral loads in internal organs, epidermal thickening, and local cytokine expression profiles (see Fig E3 in this article's Online Repository at www.jacionline.org). Differences in the aforementioned changes between *ft/ft* or *ft/ft.ma*^{wt/wt} mice and WT control mice, but not between *ft/ft* and *ft/ft.ma*^{wt/wt} mice, were statistically significant. These results indicate that the *ma* mutation does not play an important role in the susceptibility of *ft/ft* mice to VV inoculation.

VV inoculation of topically sensitized skin aggravates the features of EV in *ft/ft* mice

Topical application of OVA to the shaved unstripped back skin elicited local skin inflammation and a systemic immune response in *ft/ft* mice on a BALB/c background (see Fig E4, A–D, in this article's Online Repository at www.jacionline.org and data not shown), as previously shown in *ft/ft* mice on a mixed background.¹⁸ In contrast, topical application of OVA to the

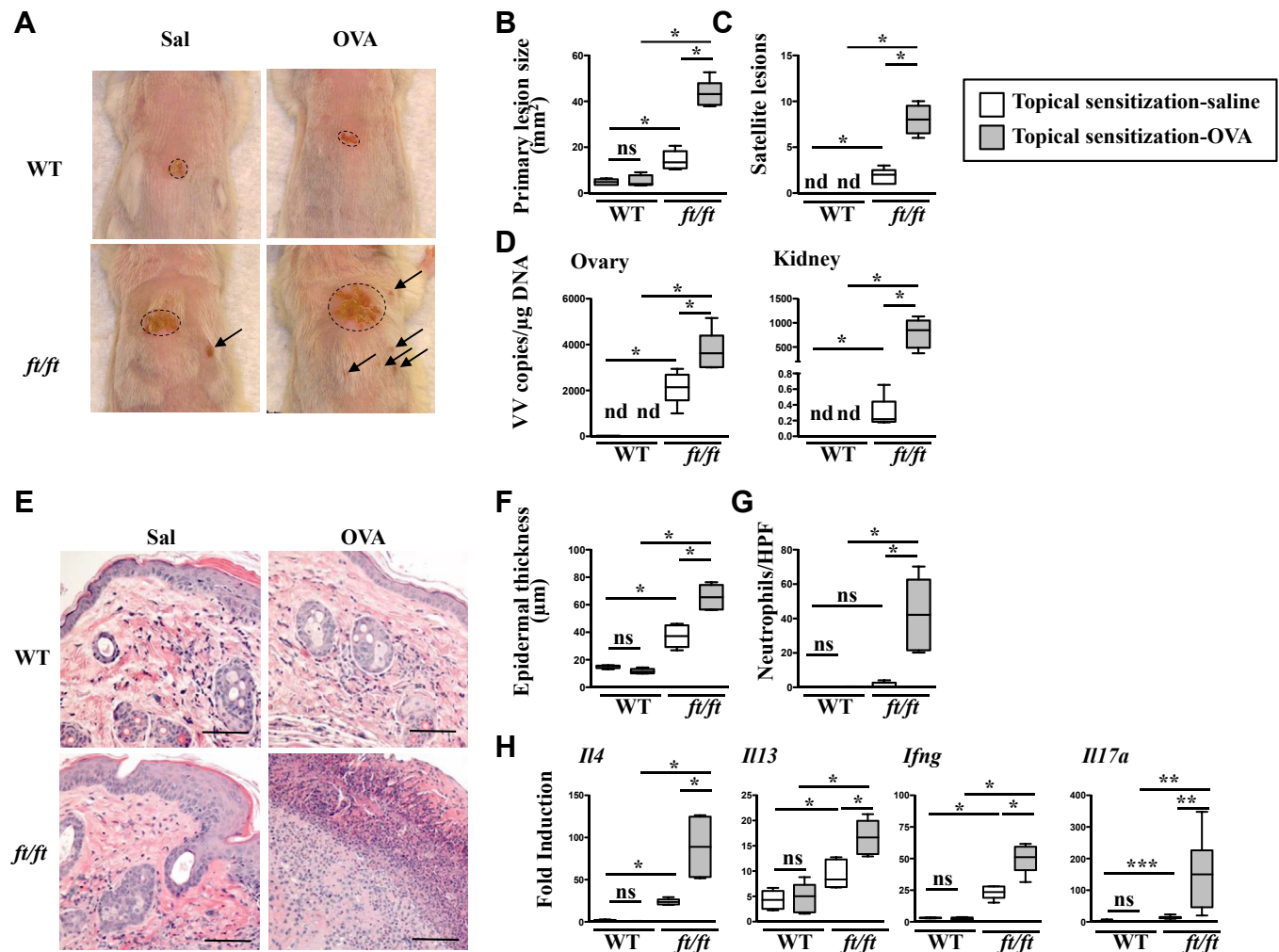


FIG 2. Exaggerated EV features in *ft/ft* mice inoculated with VV in OVA-sensitized skin. **A-C**, Gross appearance (Fig 2, A), area of primary lesions (Fig 2, B), and number of satellite lesions (Fig 2, C). *Dashed circles* indicate primary lesions. *Arrows* indicate satellite lesions. **D**, Viral load. **E-H**, H&E-stained skin sections ($\times 200$ magnification; Fig 2, E), epidermal thickness (Fig 2, F), number of neutrophils (Fig 2, G), and cytokine mRNA expression (Fig 2, H). Scale bars = 100 μ m. The line in the box indicates the median, and the whiskers represent the minimum to the maximum ($n = 5$ per group). * $P < .05$, ** $P < .01$, and *** $P < .001$. nd, Not detectable; ns, not significant; Sal, saline.

shaved unstripped back skin did not result in the development of local skin inflammation or a systemic response in WT control mice (see Fig E4, A-D, and data not shown). We tested the hypothesis that VV inoculation of *ft/ft* mice at sites of topical OVA application would result in the development of more severe features of EV. Mice were topically sensitized with OVA or saline over a 7-week period, inoculated with VV at the site of sensitization, and examined 7 days later (see Fig E4, E). *ft/ft* mice inoculated with VV at OVA-exposed sites had significantly larger primary lesions, exhibited significantly higher numbers of satellite lesions, and had significantly greater VV loads in the ovaries and kidneys compared with *ft/ft* mice inoculated with VV at saline-exposed sites or WT control mice inoculated with VV at OVA-exposed sites (Fig 2, A-D). They also had significantly greater epidermal thickness, significantly denser dermal infiltration with neutrophils, and significantly higher expression of *Il17a*, *Il4*, *Il13*, and *Ifng* mRNA (Fig 2, E-H). The size of the primary lesion, epidermal thickness, and expression of *Il17a*, *Il4*, *Il13*, and *Ifng* mRNA were comparable in WT mice inoculated

with VV in OVA- or saline-exposed skin and significantly reduced compared with those in *ft/ft* mice inoculated with VV at saline-exposed sites (Fig 2, A-C and H). WT mice inoculated with VV in OVA- or saline-exposed skin had no satellite lesions, no detectable viral loads in the internal organs, and very few neutrophils in the primary lesion (Fig 2, C-E and G). Splenocyte cytokine secretion in response to VV stimulation was similar in *ft/ft* and WT mice (data not shown). VV-specific serum IgG_{2a} levels were higher in *ft/ft* mice than in WT mice, regardless of whether the skin was sensitized with OVA or saline (see Fig E5, A, in this article's Online Repository at www.jacionline.org). These results suggest that cutaneous sensitization to topically encountered antigens in *ft/ft* mice aggravates their susceptibility to EV.

IL-17A contributes to the increased susceptibility of *ft/ft* mice to cutaneous VV inoculation

We have previously reported that treatment with anti-IL-17A attenuates EV features in WT mice inoculated with VV in

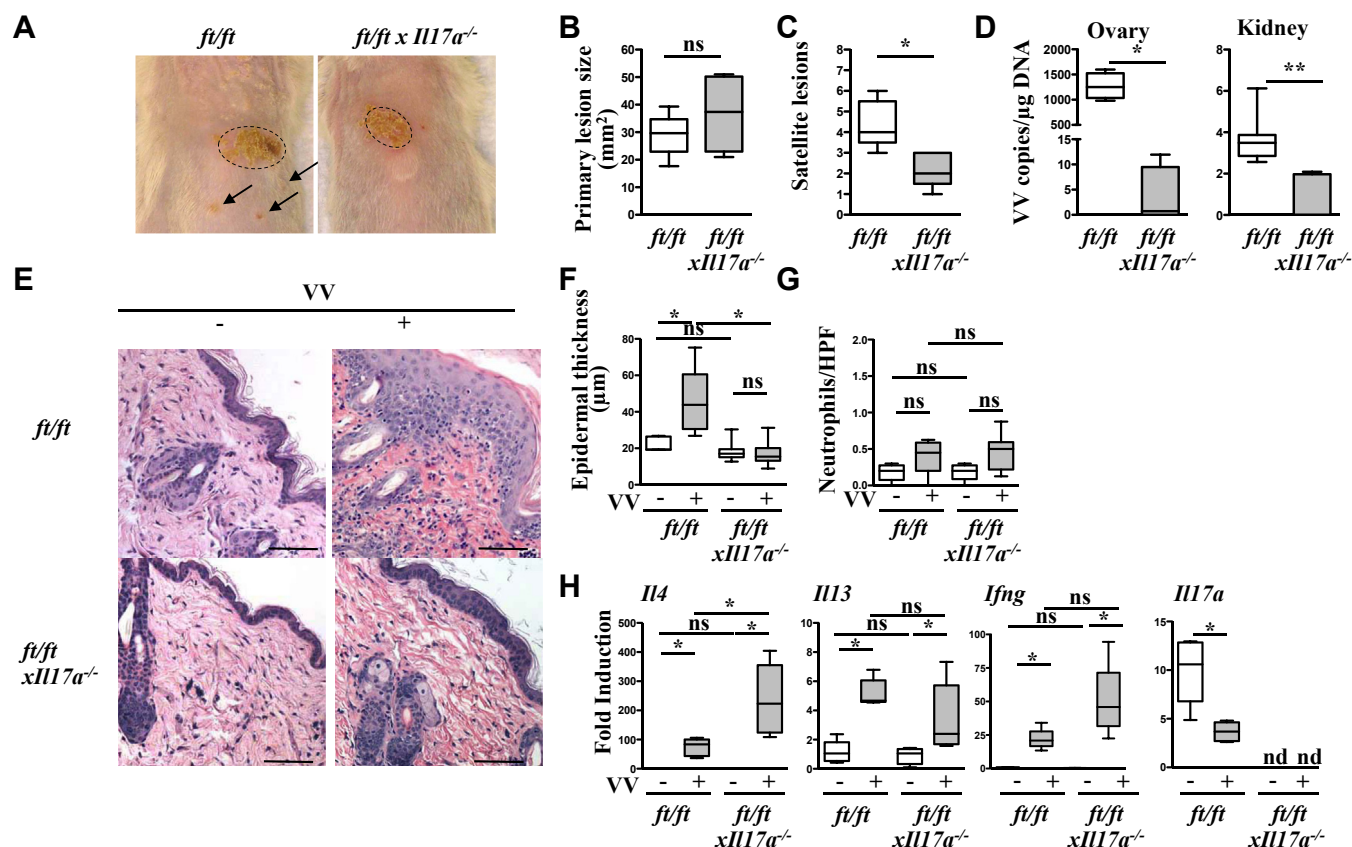


FIG 3. Absence of IL-17A attenuates EV features caused by VV inoculation in unsensitized skin of *ft/ft* mice. **A-C,** Gross appearance (Fig 3, A), area of primary lesions (Fig 3, B), and number of satellite lesions (Fig 3, C). **Dashed circles** indicate primary lesions. **Arrows** indicate satellite lesions. **D,** Viral load. **E-H,** H&E-stained skin sections (×200 magnification; Fig 3, E), epidermal thickness (Fig 3, F), number of neutrophils (Fig 3, G), and cytokine mRNA expression (Fig 3, H). Scale bars = 100 μm. The line in the box indicates the median, and the whiskers represent the minimum to the maximum (n = 5 per group). *P < .05 and **P < .01. nd, Not detectable; ns, not significant.

tape-stripped OVA-sensitized skin.³⁰ Here we tested the hypothesis that increased IL-17A expression in the skin of *ft/ft* mice contributes to their increased susceptibility to VV inoculation. We bred the *ft/ft* genotype onto the IL-17A-null background and examined responses to VV inoculation.

There was no detectable skin inflammation in 6- to 8-week-old *ft/ft x Il17a^{-/-}* mice or *ft/ft* mice. As expected, *Il17a* mRNA expression was undetectable in *ft/ft x Il17a^{-/-}* mice. After VV inoculation in unsensitized skin, *ft/ft x Il17a^{-/-}* mice had significantly fewer satellite lesions and significantly lower viral loads compared with *ft/ft* control mice, but the size of the primary lesion was comparable between the 2 groups (Fig 3, A-D). VV-inoculated *ft/ft x Il17a^{-/-}* mice had significantly less epidermal thickening than VV-inoculated *ft/ft* control mice, with no increase in dermal infiltration with neutrophils (Fig 3, E-G). However, VV-inoculated *ft/ft x Il17a^{-/-}* skin showed significantly higher levels of *Il4* mRNA expression than VV-inoculated *ft/ft* skin. Levels of *Il13* and *Ifng* mRNA in VV-inoculated skin were not significantly different between the 2 groups (Fig 3, H). VV-inoculated *ft/ft x Il17a^{-/-}* skin exhibited comparable epidermal thickness and dermal infiltration with neutrophils but significantly higher levels of *Il4*, *Il13*, and *Ifng* mRNA compared with uninfected *ft/ft x Il17a^{-/-}* skin (Fig 3, E-H). Epidermal thickness, dermal infiltration with neutrophils,

and skin *Il4*, *Il13*, and *Ifng* mRNA levels were comparable between uninfected *ft/ft* and *ft/ft x Il17a^{-/-}* mice (Fig 3, E-H). Splenocyte secretion of IL-4, IL-13, and IFN-γ in response to VV stimulation, and VV-specific serum IgG_{2a} levels were comparable in *ft/ft x Il17a^{-/-}* and *ft/ft* mice inoculated with VV (data not shown and see Fig E5, B).

We next examined the response of *ft/ft x Il17a^{-/-}* mice to VV inoculation at the site of cutaneous sensitization with OVA. After VV inoculation in skin topically sensitized with OVA, *ft/ft x Il17a^{-/-}* mice had significantly smaller primary lesions, significantly less satellite lesions, and significantly lower viral loads in internal organs compared with similarly sensitized *ft/ft* control mice (Fig 4, A-D). They also had significantly less epidermal thickening and significantly less neutrophil infiltration in the dermis; however, they expressed significantly more *Il4*, *Il13*, and *Ifng* mRNA in VV-infected skin than VV-infected OVA-sensitized skin of *ft/ft* control mice or saline-exposed *ft/ft x Il17a^{-/-}* skin (Fig 4, E-H). Splenocyte secretion of IL-4, IL-13, and IFN-γ in response to VV stimulation and VV-specific serum IgG_{2a} levels were comparable in topically sensitized or saline-exposed *ft/ft x Il17a^{-/-}* and *ft/ft* mice inoculated with VV (data not shown and see Fig E5, C). The sizes of primary lesions, numbers of satellite lesions, viral loads in internal organs, epidermal thickness, and dermal neutrophil

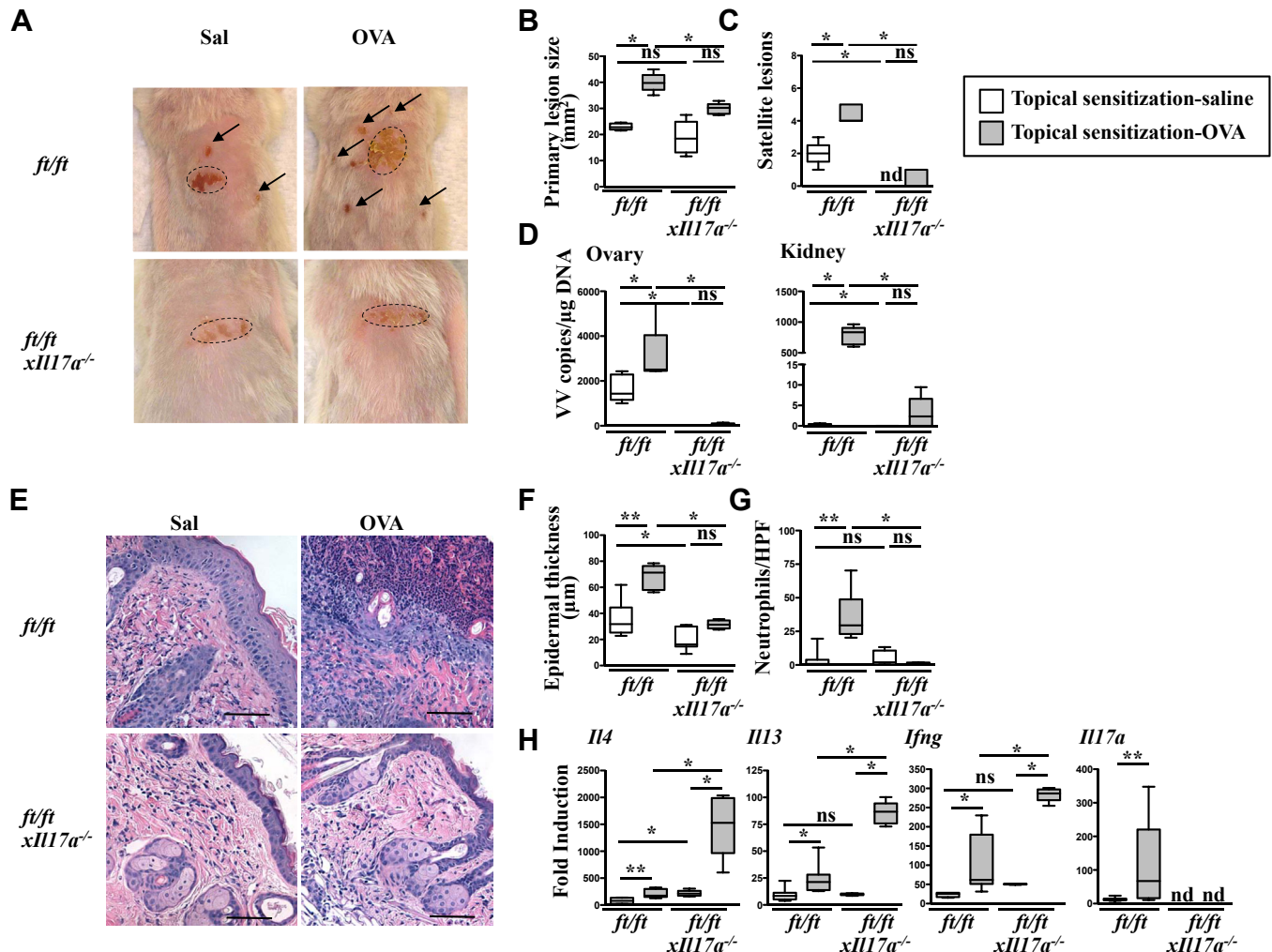


FIG 4. Absence of IL-17A attenuates EV features caused by VV inoculation in OVA-sensitized skin of *ft/ft* mice. **A-C**, Gross appearance (Fig 4, A), area of primary lesions (Fig 4, B), and number of satellite lesions (Fig 4, C). Dashed circles indicate primary lesions. Arrows indicate satellite lesions. **D**, Viral load. **E-H**, H&E-stained skin sections ($\times 200$ magnification; Fig 4, E), epidermal thickness (Fig 4, F), number of neutrophils (Fig 4, G), and cytokine mRNA expression (Fig 4, H). Scale bars = 100 μ m. The line in the box indicates the median, and the whiskers represent the minimum to the maximum ($n = 5$ per group). * $P < .05$ and ** $P < .01$. nd, Not detectable; ns, not significant; Sal, saline.

infiltration were comparable between topically sensitized or saline-exposed *ft/ftxIl17a^{-/-}* mice inoculated with VV. These results suggest that increased expression of IL-17A in the skin of *ft/ft* mice contributes to their enhanced susceptibility to cutaneous infection with VV.

DISCUSSION

We demonstrate that FLG-deficient *ft/ft* mice are more susceptible to features of EV after VV inoculation in the skin and that FLG deficiency and allergic inflammation synergize to promote VV dissemination. The increased susceptibility of *ft/ft* mice to EV depends on IL-17A.

ft/ft mice, but not WT control mice, had features of EV after VV inoculation in unsensitized skin. These included larger primary lesions at the site of cutaneous VV inoculation, higher numbers of satellite skin lesions, and greater viral dissemination to internal

organs compared with those seen in WT control mice. The increased susceptibility of *ft/ft* mice to VV dissemination, a feature of EV, is in line with the observation that *FLG* mutations predispose patients with AD to EH.¹¹ Taken together, these results suggest that FLG plays a critical role in containing viral dissemination after cutaneous infection.

The lesions in *ft/ft* mice were characterized by epidermal thickening, which was significantly greater than in WT control mice, and massive upregulation of T_H1 and T_H2 cytokine expression compared with that seen in WT control mice. The T_H1 cytokine IFN- γ and IgG_{2a} antibody play a protective role in limiting VV dissemination.³³ Splenocyte production of IFN- γ in response to VV stimulation was comparable in *ft/ft* and WT control mice, whereas VV-specific IgG_{2a} antibody levels were slightly increased in *ft/ft* mice, ruling out a role for a difference in these systemic immune responses in the increased susceptibility of *ft/ft* mice to VV. These results rather suggest that disrupted barrier

function underlies the propensity of *fl/fl* mice for VV dissemination. The increase in VV-specific IgG_{2a} antibody levels in *fl/fl* mice might be the consequence of their increased antigenic load.

Il17a mRNA expression is significantly increased in the skin of *fl/fl* mice raised under specific pathogen-free conditions.¹⁸ The mechanisms by which FLG deficiency predispose to increased IL-17A skin expression might include increased absorption of microbes from the skin, which drives the production of IL-17A–promoting cytokines, including IL-6 and IL-23.¹⁸ This is supported by the observation that IL-17A expression in the skin is attenuated in *fl/fl* mice raised under germ-free conditions (data not shown). Paradoxically, *Il17a* mRNA expression was attenuated after VV inoculation, possibly because of downregulation by the massive increase in *Il4* and *Ifng* expression.^{34,35} The increased expression of T_H1 and T_H2 cytokines in VV-inoculated skin of *fl/fl* mice was not simply a reflection of a stronger systemic antigen specific immune response in these mice. In fact, splenocytes from *fl/fl* and WT control mice secreted comparable amounts of cytokines in response to VV stimulation. One possible explanation for the local increase in T_H1 and T_H2 cytokine production in VV-inoculated skin of *fl/fl* mice could be increased cutaneous expression of molecules that promote cytokine production by infiltrating T cells. These could include thymic stromal lymphopoietin, which amplifies T_H2 cytokine expression,³⁶ and IL-12, which stimulates IFN- γ expression by T_H1 cells.³⁷ Increased dermal cellular infiltration in VV-inoculated skin sites of *fl/fl* mice might also have contributed to the increased cytokine expression at these sites.

Cutaneous antigen sensitization in *fl/fl* mice mimics skin exposure to environmental antigens in patients with AD whose barrier dysfunction allows cutaneous sensitization on exposure to antigen.^{18,38} Topical sensitization with OVA elicited a vigorous T_H2 response in the skin of *fl/fl* mice on a BALB/c background, as we had previously shown in mice on a mixed background.¹⁸ VV inoculation of *fl/fl* mice in skin sites topically sensitized with OVA exaggerated the EV features that develop in these mice, including primary lesion size, number of satellite lesions, and viral loads in internal organs, suggesting that FLG deficiency and allergic inflammation synergize to promote VV dissemination. It also resulted in more severe skin inflammation characterized by intense dermal neutrophil infiltration and an increase in *Il17a* expression compared with that seen after VV inoculation in saline-exposed or unsensitized skin from these mice. Consistent with our previous report,¹⁸ topical application of OVA to shaved unstripped WT skin did not result in development of skin inflammation and elicited minimal responses to VV inoculation. In contrast, EC sensitization of WT mice through application of OVA to tape-stripped skin, which disrupts the skin barrier, elicits a vigorous T_H2 response.²⁸ Moreover, VV inoculation in EC-sensitized tape-stripped skin of WT mice results in the development of EV features and in massive local neutrophil infiltration and *Il17a* expression.³⁰ These observations suggest that a pre-existing cutaneous inflammatory response to an unrelated antigen promotes the development of EV features and enhances the local IL-17A response when VV is inoculated in the skin.

The increased susceptibility of *fl/fl* mice to having satellite lesions and viral dissemination after VV inoculation in unsensitized skin was dependent on IL-17A. The number of satellite lesions and viral loads in internal organs in *fl/fl;Il17a^{-/-}* mice inoculated in unsensitized skin or in skin

topically sensitized with OVA were reduced to levels seen in WT control mice. T_H1 and T_H2 cytokine secretion by VV-stimulated splenocytes and serum levels of VV-specific IgG_{2a} antibody were comparable in *fl/fl;Il17a^{-/-}* and *fl/fl* mice, indicating that IL-17A played no detectable role in the systemic T_H1 and T_H2 responses of *fl/fl* mice to cutaneous inoculation with VV. However, in the absence of IL-17A, epidermal thickening was reduced and mRNA expression of *Il4* was increased in VV-inoculated skin of *fl/fl;Il17a^{-/-}* mice. These findings are consistent with previous observations that IL-17A activates keratinocytes³⁹⁻⁴¹ and downregulates T_H2 cytokine expression.³⁵ Neutrophil infiltration in VV-inoculated OVA-sensitized skin, which was observed in *fl/fl* mice, was abolished in *fl/fl;Il17a^{-/-}* mice. This finding is consistent with the role of IL-17A in driving neutrophil chemotaxis through induction of CXCL2 expression in target cells.⁴² This finding is also in agreement with our previous finding that administration of anti-IL-17A neutralizing antibody inhibits neutrophil accumulation in tape-stripped EC-sensitized skin of WT mice.³⁰ These findings suggest that IL-17A plays a critical role in the development of EV and in the local neutrophilic infiltration of VV-inoculated skin in *fl/fl* mice.

The vast majority of patients with AD with *FLG* mutations are heterozygous.⁴³ However, mice heterozygous for the *fl* mutation have no detectable skin abnormalities and do not mount an immune response to topical sensitization with antigen (our unpublished observations). Consistent with their lack of a cutaneous phenotype, *fl/+* heterozygous mice had a response to VV inoculation comparable with that seen in WT control mice (data not shown). Altogether, these findings suggest that genetic factors, environmental factors, or both operate in human subjects to modulate the effect of heterozygous *FLG* mutations in human subjects on skin biology and thereby susceptibility of VV inoculation. Patients with AD can have EV, even in the absence of active skin lesions.⁴⁴ The FLG-deficient mice we studied had no grossly visible dermatitis, yet they were susceptible to EV features after VV inoculation in the skin. This parallel with the human AD phenotype further supports the validity of using FLG-deficient mice as a model for the study of EV. The protective effect of IL-17A deficiency in these mice suggests that neutralization of IL-17A might be an effective therapy for EV in FLG-deficient patients with AD.

We thank Dr Hans Oettgen for scientific discussion and reading the manuscript. We thank Dr Denise Babineau for discussion about the statistical analysis of our experimental data.

Clinical implications: IL-17A blockade might prevent the development of EV.

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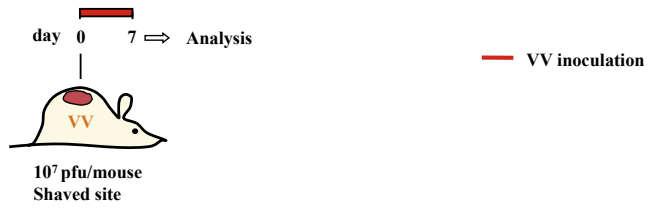


FIG E1. Experimental design of cutaneous VV inoculation using *ft/ft* mice. Back skin of mice was shaved and inoculated with 10^7 plaque-forming units (*pfu*) of VV per mouse by means of skin scarification. Mice were killed after 7 days of VV inoculation and examined for clinical responses, viral load, serum VV antibody levels, and cytokine production, as previously described.^{E1}

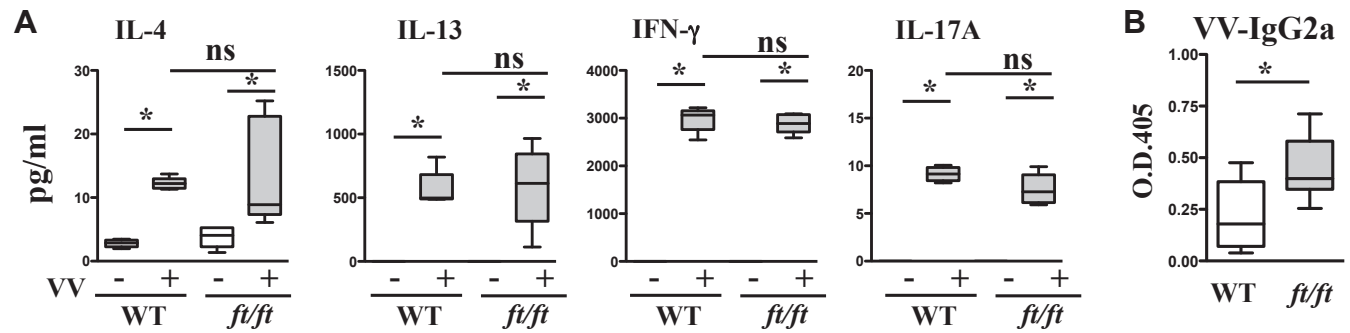


FIG E2. VV-specific systemic immune responses in *ft/ft* mice cutaneously inoculated with VV. **A**, VV-specific cytokine production by splenocytes after stimulation with VV-infected A20 cells. **B**, Serum VV-specific IgG_{2a} antibody levels. The line in the box indicates the median, and the whiskers represent the minimum to the maximum (n = 5 per group). **P* < .05. ns, Not significant.

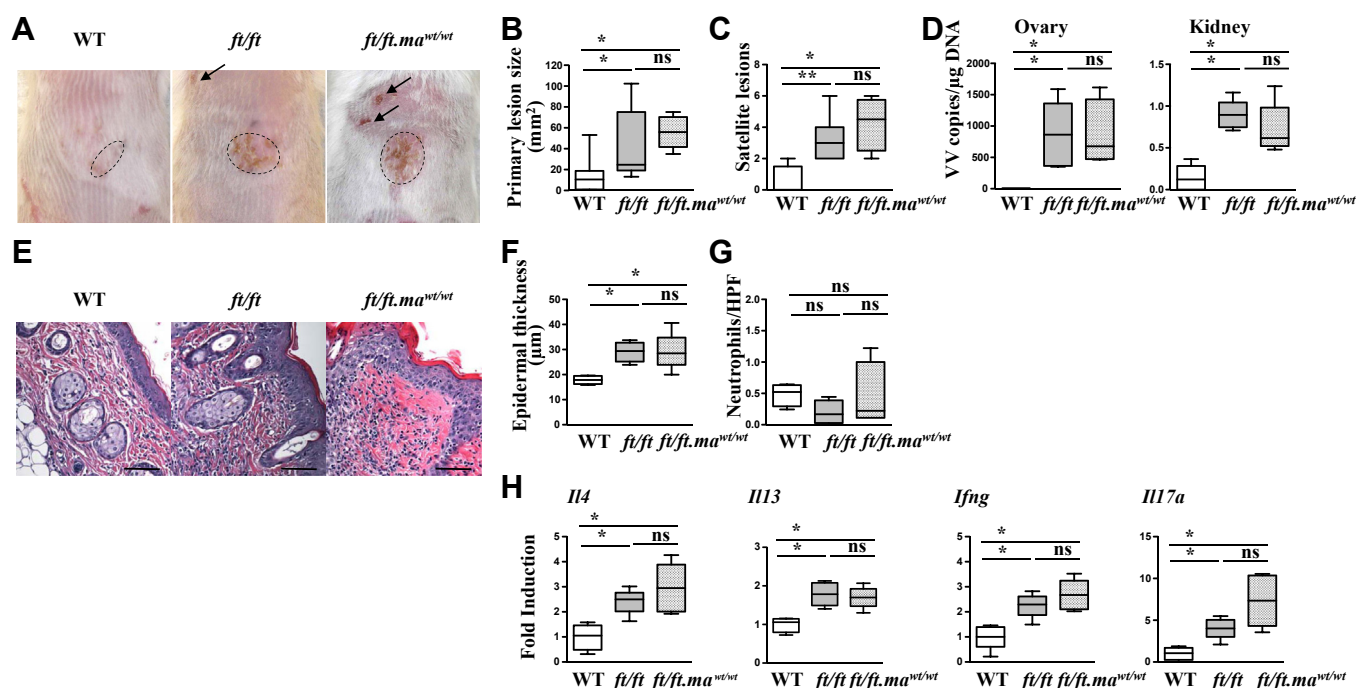


FIG E3. EV features in *ft/ft* mice are not influenced by the *ma* mutation. **A-C**, Primary and satellite lesions in *ft/ft* mice homozygous for the *ma* mutation (*ft/ft*) or without the *ma* mutation (*ft/ft.ma^{wt/wt}*) and WT control mice inoculated with VV in shaved back skin: gross appearance (Fig E3, A), area of primary lesions (Fig E3, B), and number of satellite lesions (Fig E3, C). Dashed circles indicate primary lesions. Arrows indicate satellite lesions. **D**, Viral load. **E-G**, H&E-stained skin sections ($\times 200$ magnification; Fig E3, E), epidermal thickness (Fig E3, F), number of neutrophils (Fig E3, G), and cytokine mRNA expression as fold induction relative to VV-inoculated WT skin (Fig E3, H). Scale bars = 100 μ m. The line in the box indicates the median, and the whiskers represent the minimum to the maximum ($n = 5$ per group). * $P < .05$ and ** $P < .01$. ns, Not significant.

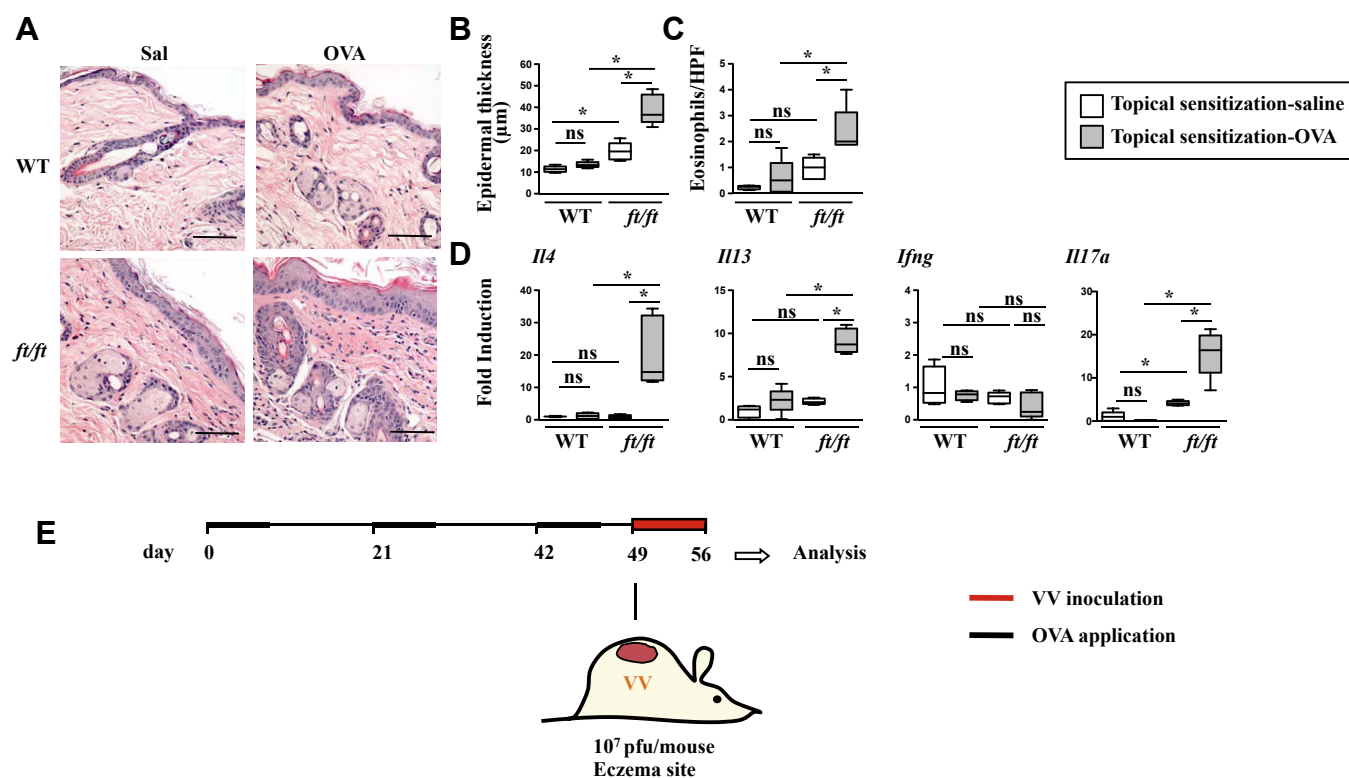


FIG E4. Histology and cytokine expression of skin topically sensitized with OVA in *ft/ft* mice. **A**, H&E-stained skin sections. **B**, Epidermal thickness. **C**, Numbers of eosinophils. **D**, Cytokine mRNA expression expressed as fold induction relative to saline-exposed WT skin. Scale bars = 100 μ m. **E**, Experimental plan of VV inoculation in topically sensitized skin. Mice were shaved and topically sensitized with OVA or saline for three 1-week-long cycles with 2-week rest intervals (total of 7 weeks),^{E2} followed by inoculation of 10⁷ plaque-forming units (*pfu*) per mouse by means of skin scarification. Mice were killed after 7 days of VV inoculation and examined for clinical responses, viral load, serum levels of VV antibodies, and cytokine production, as previously described.^{E1} The line in the box indicates the median, and the whiskers represent the minimum to the maximum ($n = 5$ per group). * $P < .05$. ns, Not significant; Sal, saline.

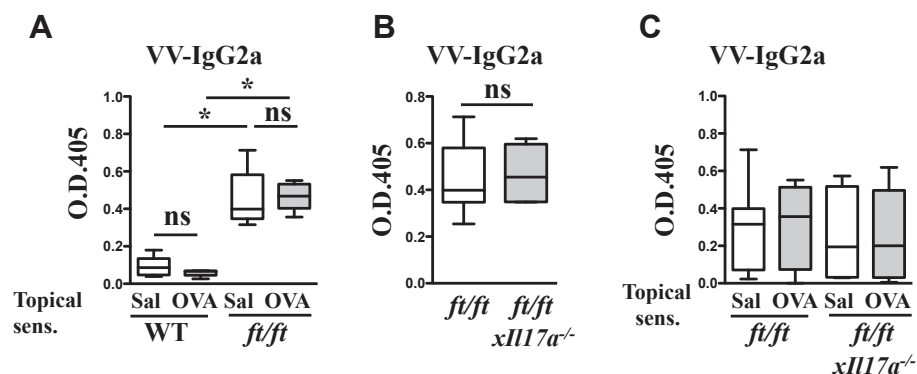


FIG E5. VV-specific IgG_{2a} antibody response to cutaneous VV inoculation. **A**, VV-specific IgG_{2a} levels in *ft/ft* and WT control mice topically sensitized with OVA or saline. **B** and **C**, VV-specific IgG_{2a} levels in *ft/ft* and *ft/ftxIl17a^{-/-}* mice inoculated in unsensitized skin (Fig E5, **B**) or in skin topically sensitized with OVA or saline (Fig E5, **C**). The line in the box indicates the median, and the whiskers represent the minimum to the maximum (n = 5 per group). **P* < .05. ns, Not significant; Sal, saline.