

# Cytokine Milieu of Atopic Dermatitis Skin Subverts the Innate Immune Response to Vaccinia Virus

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## Summary

Atopic dermatitis (AD) is associated with eczema vaccinatum (EV), a disseminated viral skin infection that follows inoculation with vaccinia virus (VV). This study examined whether AD skin can control VV replication, and the role of IL-4 and IL-13 in modulating the human cathelicidin LL-37, an antimicrobial peptide that kills VV. AD skin exhibited increased VV replication and decreased LL-37 expression compared to normal or psoriasis skin. IL-4/IL-13 enhanced VV replication while downregulating LL-37 in VV-stimulated keratinocytes. Neutralizing IL-4/IL-13 in AD skin augmented LL-37 and inhibited VV replication. Cathelicidins were induced via toll-like receptor-3 and were inhibited by IL-4/IL-13 through STAT-6. Skin from cathelicidin-deficient mice exhibited reduced ability to control VV replication. Exogenous LL-37 controlled vaccinia viral replication in infected keratinocytes and AD skin explants. The current study demonstrates that Th2 cytokines enhance VV replication in AD skin by subverting the innate immune response against VV in a STAT-6-dependent manner.

## Introduction

In 1980, the World Health Organization (WHO) declared that endemic smallpox had been eradicated (World Health Organization, 1980). The events of September 11, 2001, and the subsequent bioterrorist attacks with anthrax have led to an unprecedented degree of concern over potential future bioterrorist attacks with other select agents such as smallpox and the plague (Lane et al., 2001). It is estimated that 119 million residents of the U.S. have been born since the smallpox vaccination was discontinued. Such individuals are susceptible to smallpox infection, an epidemic of which could result in catastrophic numbers of death and disease (Bicknell,

2002). This possibility has led to a debate over reinstating voluntary vaccinations against smallpox (Bicknell, 2002; Fauci, 2002; Drazen, 2002).

Individuals with atopic dermatitis (AD) are excluded from voluntary smallpox vaccination due to their predisposition to develop eczema vaccinatum (EV). The potential impact of this recommendation was seen recently as up to 34% of military personnel deferred voluntary smallpox vaccination, with various contraindications with skin conditions being the main cause for deferral (Grabenstein and Winkenwerder, 2003).

Approximately 17% of children are diagnosed with AD (Leung et al., 2004; Leung and Bieber, 2003), and, according to the current Centers for Disease Control guidelines, these children and those in close contact with them should not be vaccinated (Rotz et al., 2001). It is not understood why AD patients are susceptible to developing EV. This prompted the National Institutes of Health to fund the Atopic Dermatitis Vaccinia Network to further investigate this important public health concern (<http://www.NIAID.NIH.gov/contract/archive/RFP0406.pdf>).

Antimicrobial peptides (AMPs) are an integral component of the innate immune response that determines susceptibility to infection (Ong et al., 2002; Dorschner et al., 2001). Recently, our laboratory demonstrated that the cathelicidin family, i.e., LL-37, of AMPs exhibit antiviral activity against purified VV (Howell et al., 2004). In an effort to understand the potential mechanisms of EV, the current study was carried out to evaluate the ability of cultured AD skin, as compared to normal or psoriasis skin, to control the replication of VV, as well as the effect of IL-4 and IL-13, which are overexpressed in AD skin (Hamid et al., 1994), on the cathelicidin response to VV infection.

## Results

### VV Replication in AD Skin Explant Cultures

Figure 1A demonstrates that VV replication was enhanced in biopsies from the uninvolved skin of AD patients (mean: 1241 ± 485 ng VV/ng GAPDH) compared to normal (mean: 312 ± 44;  $p < 0.05$ ) and psoriasis (mean: 281 ± 59;  $p < 0.05$ ) skin. This finding was confirmed at the protein level by immunofluorescent staining for an early viral protein found in replicating VV (Yuwen et al., 1993) (Figure 1B). Similar levels of VV staining were observed in the stratum corneum of each subject group; however, skin from AD patients exhibited significantly more intense staining for VV in the basal keratinocytes than did skin from normal ( $p < 0.05$ ) and psoriasis ( $p < 0.05$ ) patients (Figure 1C).

AD skin is characterized by the overexpression of IL-4 and IL-13 (Hamid et al., 1994). We therefore examined the direct effects of these Th2 cytokines on keratinocytes stimulated with VV (0.1 pfu/cell). Addition of IL-4 and IL-13 significantly augmented VV replication (mean: 21,280 ± 4,814 ng VV/ng GAPDH;  $p < 0.001$ ) compared to media alone (mean: 12,760 ± 1,263) (Figure 2).

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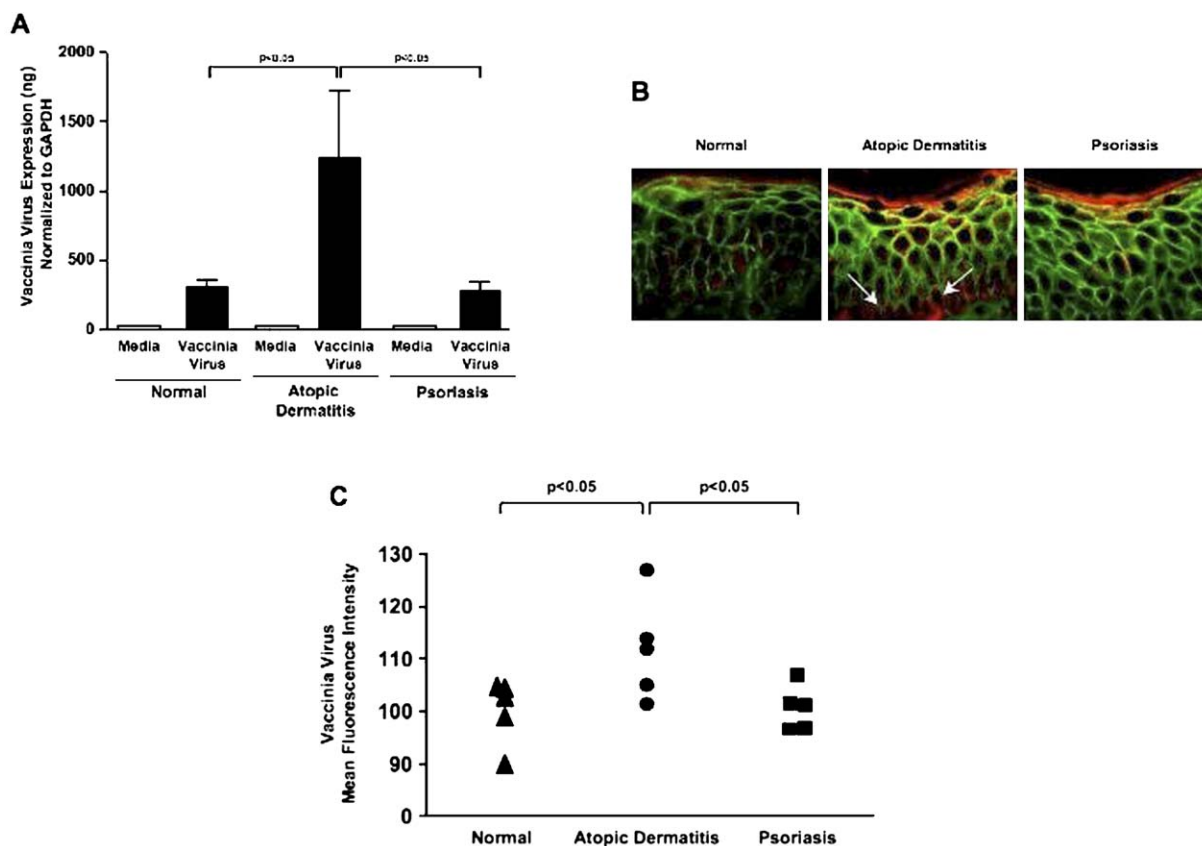


Figure 1. Increased Vaccinia Virus Gene Expression in AD Skin

(A) RNA was isolated from nonlesional skin biopsies of normal ( $n = 7$ ), AD ( $n = 7$ ), and psoriasis ( $n = 6$ ) patients stimulated with either media or vaccinia virus for 24 hr and was analyzed for vaccinia virus gene expression by real-time RT-PCR. Data are expressed as the mean  $\pm$  SEM.

(B) Nonlesional skin biopsies from normal ( $n = 5$ ), AD ( $n = 5$ ), and psoriasis ( $n = 5$ ) patients were stimulated with media or vaccinia virus for 24 hr and were stained for E3L, an early vaccinia virus protein. Arrows point to intense vaccinia virus staining in basal keratinocytes of a representative skin biopsy from each study group.

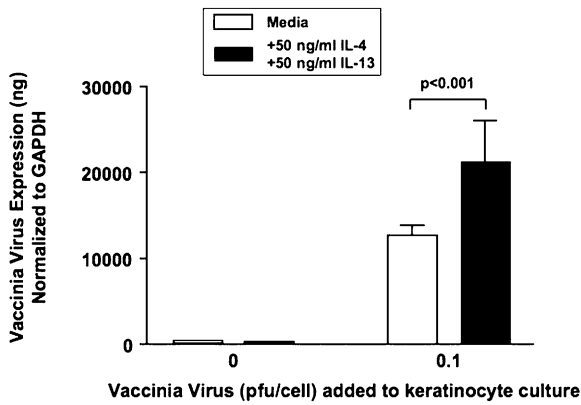
(C) The mean fluorescence intensity is shown for vaccinia virus expression in the basal keratinocytes of each biopsy.

### Essential Role of Cathelicidins in Controlling VV Replication

To investigate the role of skin cathelicidins in the innate immune response to VV, the gene expression of LL-37 was evaluated by real-time PCR in skin biopsies from AD patients, psoriasis patients, and normal subjects that were stimulated with VV. Stimulation with VV induced significantly higher levels of LL-37 in normal (mean:  $28.08 \pm 6.89$  ng LL-37/ng GAPDH;  $p < 0.05$ ) and psoriasis (mean:  $30.56 \pm 6.65$ ;  $p < 0.05$ ) skin compared to the media-stimulated biopsies from the same donors (normal mean:  $10.60 \pm 2.39$ ; psoriasis mean:  $11.68 \pm 1.89$ ) (Figure 3A). In contrast, stimulation with VV failed to significantly induce LL-37 expression in skin biopsies from AD patients (media mean:  $10.93 \pm 3.63$ ; vaccinia mean:  $11.22 \pm 3.90$ ). This was confirmed at the protein level by immunostaining for LL-37 (Figure 3B). VV-stimulated, nonlesional skin from normal and psoriasis patients exhibited more intense staining for LL-37 than did skin from AD patients. The composite data for LL-37 immunostaining in all samples are shown in Figure 3C. The intensity of LL-37 staining in vaccinia-stimulated, nonlesional AD skin was significantly lower than that for normal ( $p < 0.05$ ) and psoriasis ( $p < 0.01$ ) skin. The basal cell layer of

AD skin, where VV replication was greatest (see Figure 1B), was relatively devoid of LL-37 staining, and it exhibited much less staining than normal and psoriasis skin.

Human keratinocyte cultures were infected with VV for 6 hr and then treated with exogenous LL-37 to determine whether the virus replication could be halted with physiologic concentrations of LL-37. Figure 4A demonstrates that concentrations of LL-37 as low as  $25 \mu\text{M}$  significantly ( $p < 0.05$ ) reduced the levels of VV gene expression in previously infected keratinocytes. This was further investigated by stimulating skin biopsies from AD patients with  $2 \times 10^5$  pfu vaccinia for 6 hr, followed by the addition of exogenous LL-37. Treatment with LL-37 significantly ( $p < 0.05$ ) reduced viral replication in AD skin biopsies (mean:  $8.33 \pm 1.85$  ng VV/ng GAPDH) compared to VV alone ( $35.15 \pm 4.64$  ng VV/ng GAPDH) (Figure 4B). Additionally, we used mice deficient in CRAMP, the murine homolog of LL-37, to further investigate the relationship between cathelicidins and VV replication in the skin. Significantly higher levels of VV replication were observed in skin biopsies from CRAMP knockout mice (BALB/c background) ( $21.04 \pm 9.67$  ng VV/ng GAPDH;  $p < 0.05$ ) compared to skin biopsies from wild-type BALB/c mice ( $1.09 \pm 0.22$ ) (Figure 4C).



**Figure 2. IL-4 and IL-13 Augment Vaccinia Virus Replication**  
Human keratinocytes were stimulated with vaccinia virus in the presence and absence of IL-4 and IL-13 for 24 hr. RNA was collected from the cells, and the levels of vaccinia virus were evaluated by real-time RT-PCR. Data are expressed as the mean  $\pm$  SEM and are representative of three experiments.

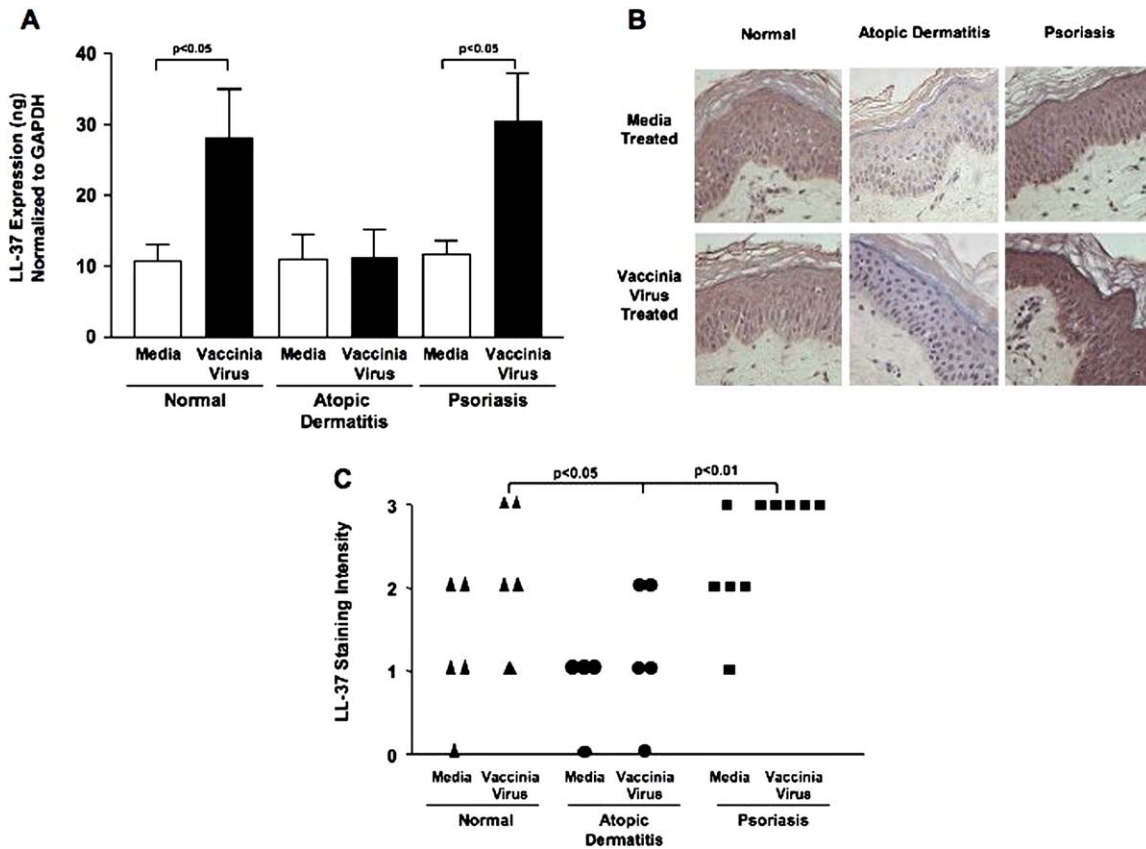
**Induction of LL-37 through TLR-3**

The mechanism by which VV induces LL-37 is not known. Since VV is known to target the toll-like recep-

tors (TLR) (Stack et al., 2005), we investigated whether signaling through TLR-3 with polyinosinic-polycytidylic acid (Poly I:C) would induce LL-37 in human keratinocytes. Stimulation with 250  $\mu$ g/ml Poly I:C significantly induced LL-37 expression ( $2.50 \pm 0.19$  ng LL-37/ng GAPDH;  $p < 0.01$ ) compared to media alone ( $0.82 \pm 0.05$ ). Levels were similar to those observed in keratinocytes stimulated with 0.1 pfu/cell VV ( $2.03 \pm 0.29$ ) (Figure 5A). These data are representative of three independent experiments. To further investigate the role of TLR-3, skin biopsies from mice deficient in TLR-3 were inoculated with VV. Levels of CRAMP were found to be significantly decreased in TLR-3 knockout mice ( $0.62 \pm 0.08$  ng CRAMP/ng GAPDH;  $p < 0.05$ ) compared to wild-type mice ( $2.84 \pm 0.78$ ) (Figure 5B).

**Th2 Cytokines Downregulate VV-Induced LL-37 Expression through STAT-6**

To further investigate why VV infection fails to significantly induce LL-37 expression in AD, human keratinocytes were cultured in the presence of Th2 cytokines. The addition of IL-4 and IL-13 prior to VV stimulation (0.1 pfu/cell) reduced the levels of LL-37 by over 75% (media alone mean:  $1.29 \pm 0.13$  ng LL-37/ng GAPDH; IL-4/IL-13-treated mean:  $0.16 \pm 0.01$ ) (Figure 6). We next



**Figure 3. Vaccinia Virus Fails to Induce LL-37 Expression in AD**  
(A) RNA was isolated from nonlesional skin biopsies of normal (n = 7), AD (n = 7), and psoriasis (n = 6) patients stimulated with either media or vaccinia virus for 24 hr and was analyzed for LL-37 gene expression by real-time RT-PCR. Data are expressed as the mean  $\pm$  SEM.  
(B) Nonlesional skin biopsies from normal (n = 5), AD (n = 5), and psoriasis (n = 5) patients were stimulated with media or vaccinia virus for 24 hr and were stained for LL-37 immunohistochemistry.  
(C) The intensity of the LL-37 immunostaining for each subject was visually scored on a scale from 0 to 3, with 0 indicating no staining and 3 indicating the most intense staining.

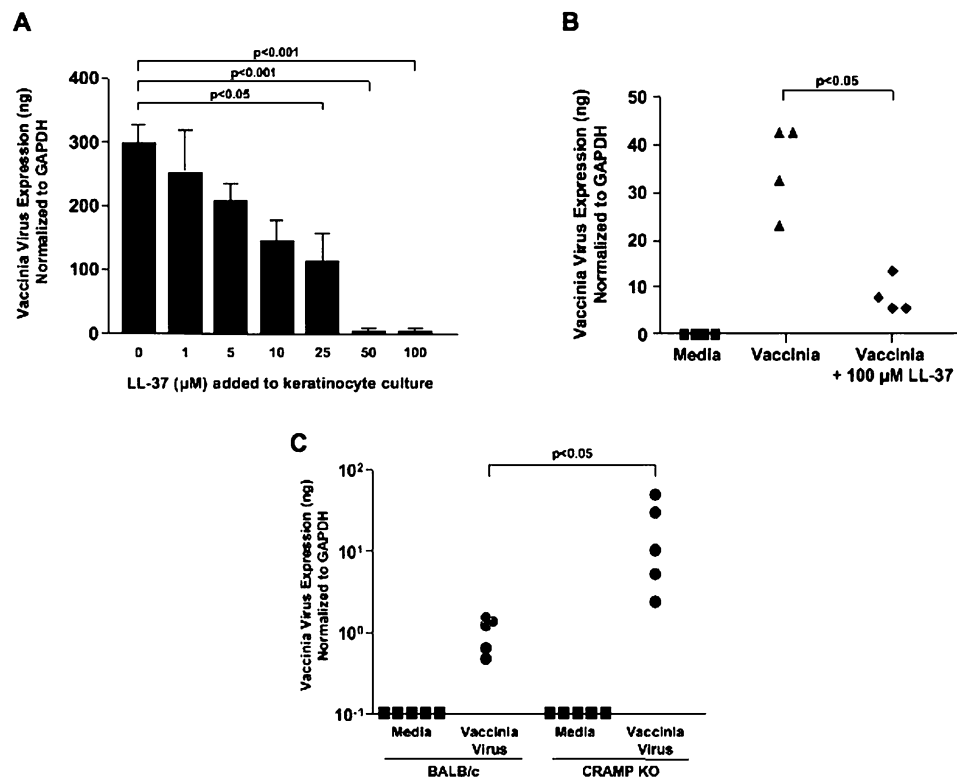


Figure 4. Essential Role of Cathelicidins in Controlling Vaccinia Virus Replication in the Skin

(A) Human keratinocytes were infected with 0.05 pfu/cell vaccinia virus for 6 hr and then treated with physiologic concentrations of LL-37 for an additional 18 hr. RNA was isolated from the cells, and the levels of vaccinia virus gene expression were evaluated by real-time RT-PCR. Data are expressed as the mean  $\pm$  SEM and are representative of three experiments. (B) Skin biopsies from AD patients ( $n = 4$ ) were stimulated with  $2 \times 10^5$  pfu vaccinia virus for 6 hr and then treated with 100  $\mu$ M LL-37 for an additional 18 hr. RNA was isolated, and the levels of vaccinia virus gene expression were evaluated by real-time RT-PCR. (C) Skin biopsies from BALB/c ( $n = 5$ ) and CRAMP knockout ( $n = 5$ ) mice were stimulated with  $2 \times 10^5$  pfu vaccinia virus for 24 hr and evaluated for vaccinia virus gene expression. RNA was collected from the tissue, and the levels of vaccinia virus were evaluated by real-time RT-PCR.

investigated whether the defective anti-VV immune response in AD skin could be reversed by the use of neutralizing antibodies to IL-4 and IL-13. Nonlesional skin biopsies were collected from AD patients and stimulated with VV in the presence and absence of anti-IL-4/anti-IL-13. Figure 7A demonstrates that neutralization

of IL-4 and IL-13 reduces the viral load by more than 75%—from  $30,740 \pm 8,091$  ng VV/ng GAPDH to  $7,334 \pm 3,325$  ( $p < 0.05$ ). Additionally, LL-37 expression was significantly increased in AD skin treated with anti-IL-4/anti-IL-13 prior to VV stimulation (mean:  $1531.5 \pm 329.3$ ;  $p < 0.001$ ) compared to AD skin stimulated with

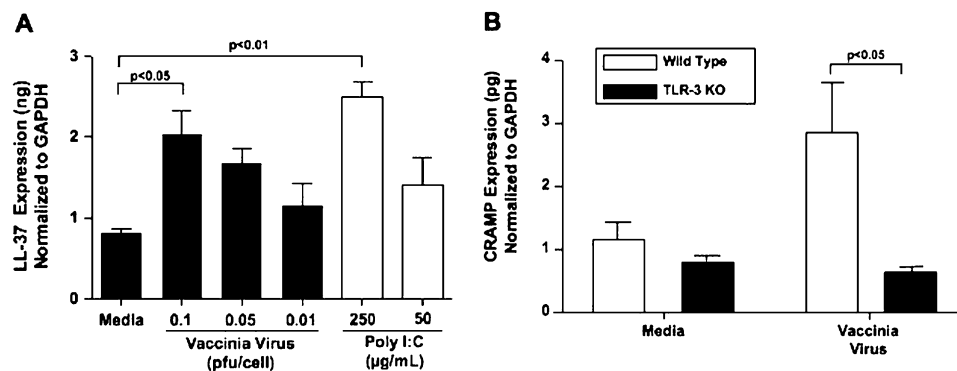
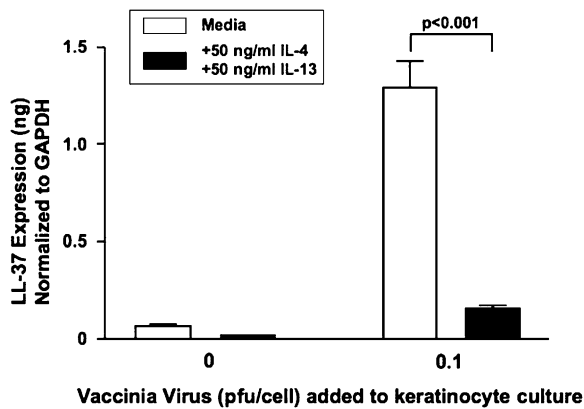


Figure 5. Vaccinia Virus Induces LL-37 by a TLR-3-Mediated Mechanism

(A) Human keratinocytes were stimulated with either vaccinia virus or Poly I:C for 24 hr. RNA was collected from the cells, and the levels of LL-37 were evaluated by real-time RT-PCR. Data are expressed as the mean  $\pm$  SEM and are representative of three experiments. (B) Skin biopsies from wild-type ( $n = 6$ ) and TLR-3 knockout mice ( $n = 6$ ) were stimulated with  $2 \times 10^5$  pfu vaccinia virus for 24 hr. RNA was isolated from the skin and analyzed for CRAMP by real-time RT-PCR. Data are expressed as the mean  $\pm$  SEM.



**Figure 6.** IL-4 and IL-13 Inhibit Vaccinia Virus-Induced LL-37  
Human keratinocytes were stimulated with vaccinia virus in the presence and absence of IL-4 and IL-13 for 24 hr. RNA was collected from the cells, and the levels of LL-37 were evaluated by real-time RT-PCR. Data are expressed as the mean  $\pm$  SEM and are representative of three experiments.

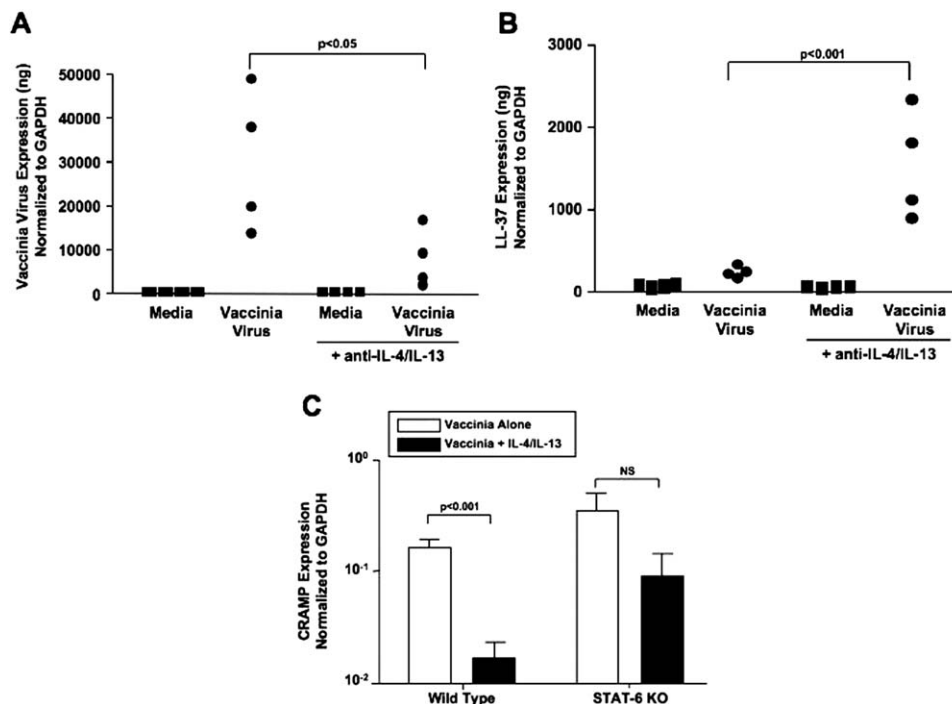
VV alone (mean:  $202.9 \pm 20.1$ ) (Figure 7B). In contrast, VV and LL-37 gene expression was not altered in normal skin biopsies by the addition of anti-IL-4/anti-IL-13 (Figure S1; see the Supplemental Data available with this article online). Since interferons (IFN) play an important role in the antiviral response, we investigated whether treatment with anti-IL-4/anti-IL-13 would increase IFN expression in AD skin. Treatment of AD skin

with anti-IL-4/anti-IL-13 prior to VV stimulation had no effect on the levels of IFN- $\gamma$  ( $43.26 \pm 35.32$  ng/ng GAPDH), IFN- $\alpha 1$  ( $60.93 \pm 40.49$  ng/ng GAPDH), or IFN- $\beta 1$  ( $31.15 \pm 20.29$  ng/ng GAPDH) compared to VV stimulation alone (IFN- $\gamma$ :  $35.78 \pm 25.04$ ; IFN- $\alpha 1$ :  $105.90 \pm 58.50$ ; IFN- $\beta 1$ :  $50.77 \pm 26.01$ ) (Figure S2).

Skin biopsies from wild-type and signal transducer and activator of transcription (STAT)-6-deficient mice were stimulated with VV in the presence and absence of IL-4 and IL-13. Wild-type skin treated with IL-4 and IL-13 prior to VV stimulation exhibited significantly lower levels of CRAMP ( $0.02 \pm 0.01$  ng CRAMP/ng GAPDH;  $p < 0.05$ ), the murine homolog to LL-37, compared to stimulation with VV alone ( $0.16 \pm 0.03$ ). In contrast, CRAMP levels were not affected by IL-4 and IL-13 in the skin of STAT-6-deficient mice (VV alone:  $0.34 \pm 0.16$ ; VV + IL-4/IL-13:  $0.09 \pm 0.06$ ) (Figure 7C).

## Discussion

Since there are currently no effective antiviral agents that can be used to treat smallpox infection, inoculation with VV remains the most effective method by which to protect individuals from smallpox infection. Understanding the mechanism(s) that results in poor control of VV in AD skin is critical to the development of new approaches for control of disseminated viral infections such as EV and may provide insight into why specific populations of humans are susceptible to adverse reactions after smallpox vaccination. At this time, it is not



**Figure 7.** IL-4 and IL-13 Inhibit Cathelicidin Expression through STAT-6 and Can Be Reversed by Neutralizing Antibodies  
(A and B) Nonlesional skin from AD ( $n = 4$ ) patients was preincubated with neutralizing antibodies to IL-4 and IL-13 versus control for 24 hr and then stimulated with vaccinia virus for an additional 24 hr. These biopsies were then analyzed for (A) vaccinia virus and (B) LL-37 mRNA expression by real-time RT-PCR.  
(C) Skin biopsies from wild-type ( $n = 5$ ) and STAT-6 knockout mice ( $n = 5$ ) were incubated with IL-4 and IL-13 for 24 hr and then stimulated with vaccinia virus for an additional 24 hr. RNA was isolated from the skin and analyzed for CRAMP by real-time RT-PCR. NS indicates no significant difference. Data are expressed as the mean  $\pm$  SEM.

ethical to subject human subjects with AD to the smallpox vaccine. Therefore, cultured human skin is a valuable approach for insights into mechanisms by which VV infection causes EV.

To our knowledge, our current study is the first to demonstrate that the skin of AD patients supports VV growth to a greater extent than normal or psoriasis skin. Increased levels of VV in AD skin were confirmed by analyzing both gene expression of the DNA-dependent RNA polymerase, which is essential for viral replication (Amegadzie et al., 1991), and immunofluorescent staining for a double-stranded RNA binding protein (Yuwen et al., 1993) expressed in the infectious cycle of VV. We hypothesized that increased growth of VV in AD skin results in the development of disseminated viral infection clinically manifested as EV.

AMPs are an integral component of the innate immune response and are one of the first lines of defense against viral pathogens. We have previously shown that members of the cathelicidin family of AMPs (i.e., LL-37), but not human  $\alpha$  defensin or human  $\beta$  defensins-1 or -2, possess antiviral activity against VV (Howell et al., 2004). LL-37 is produced by keratinocytes (Frohm et al., 1997) and is induced in response to inflammatory stimuli (Erdag and Morgan, 2002). We therefore investigated the levels of LL-37 in AD skin to determine whether increased VV replication corresponded with decreased AMP expression. After stimulation with VV, skin biopsies from normal subjects and psoriasis patients expressed increased levels of LL-37. In contrast, levels of LL-37 in AD skin remained unchanged after VV stimulation. This observation is important since LL-37 exhibits antiviral activities, and it explains, in part, the increased growth of VV observed in AD skin. The importance of cathelicidins in skin innate immune responses to VV is strongly supported by our current observation that mice deficient in CRAMP, the murine homolog to LL-37, exhibit higher levels of VV replication after inoculation than do their wild-type controls. Additionally, we demonstrate that exogenous addition of LL-37 inhibits VV replication in previously infected AD skin biopsies. This was further extended to demonstrate that concentrations of LL-37 as low as 25  $\mu$ M were able to inhibit VV replication in previously infected keratinocytes. Since psoriatic skin can contain up to 1605  $\mu$ M LL-37 (Ong et al., 2002), this demonstrates that physiologic concentrations of LL-37 are effective at controlling VV replication. This latter observation is particularly important since VV targets the basal keratinocytes of AD patients, which corresponds to the region of AD skin most deficient in LL-37.

It is not currently known how VV induces LL-37; therefore, we extended our studies to demonstrate that VV induces LL-37 through TLR-3. This was demonstrated by two approaches: first, by showing that the TLR-3 agonist, Poly I:C, induces LL-37 to the same levels as VV in cultured human keratinocytes. Second, we demonstrate that VV does not induce CRAMP in TLR-3 knockout mice.

Uninvolved AD skin is characterized by overexpression of the Th2 cytokines IL-4 and IL-13, while it is relatively devoid of the Th1 cytokines IL-12 and IFN- $\gamma$  (Ong et al., 2002; Nomura et al., 2003). To our knowledge, our current study is the first to demonstrate that IL-4 and IL-13 augment VV replication in human keratinocytes,

as evidenced by increased levels of VV gene expression. It has previously been shown, by using knockout mice, that a deficiency in IFN- $\gamma$  or IL-12 increases the susceptibility to VV infection, whereas viral replication was impaired in IL-4 knockout mice (van den Broek et al., 2000). The important role of IL-4 in pox infections was further demonstrated by Jackson et al. (2001). Mice genetically resistant to mousepox were infected with IL-4-expressing *Ectromelia* virus (mousepox). These mice developed symptoms of acute mousepox and significant mortality due to mousepox.

Since keratinocytes are the primary source of AMPs in the epidermis, we investigated the immunomodulatory effect of IL-4 and IL-13 on the expression of LL-37 in human keratinocytes stimulated with VV. In this study, we demonstrated that the addition of IL-4 and IL-13 to keratinocytes inhibits the induction of LL-37. Thus, our current study implicates the expression of IL-4 and IL-13 in AD as the cause of deficient AMP production, and increased VV replication that could contribute to the development of EV in AD skin. To provide direct support for this hypothesis, we demonstrated that inhibiting the actions of IL-4 and IL-13, with neutralizing antibodies, allows AD skin to act similarly to normal skin; i.e., VV replication was reduced and LL-37 expression was increased. Importantly, these data provide a rationale that neutralization of Th2 cytokine action in AD skin may be considered to be a therapeutic approach to boost innate immunity in AD skin and allow these individuals to be vaccinated against smallpox.

Th2 cytokines have previously been shown to act through STAT-6 (Takeda et al., 1996a, 1996b), which transcriptionally inhibits NF- $\kappa$ B binding to consensus target sequences and the subsequent expression of innate immune response genes (Ohmori and Hamilton, 2000). Since the expression of cathelicidins can be influenced at several levels, including posttranslational events such as enzymatic activation of the precursor protein and release of the peptide from intracellular granules, it was of interest to determine at what level cathelicidin is regulated in VV-infected skin. The current data show that both transcript abundance and cathelicidin immunoreactivity are induced by VV. Furthermore, by using STAT-6-deficient mice, we demonstrate that IL-4 and IL-13 signal through STAT-6 to inhibit the expression of the cathelicidin transcript. As this process correlates with antiviral activity and a NF- $\kappa$ B binding site has been identified in the human cathelicidin promoter (Pestonjamas et al., 2001), it is likely that control of cathelicidin expression in this system occurs, at least in part, at the transcriptional level.

Since there is a significant life-long risk associated with vaccination of AD patients, it is important to identify potential therapeutic agents that would allow for the development of an immune response while reducing the potential for adverse events. Previous data from our laboratory combined with this study would suggest the use of LL-37 as an antiviral agent (Howell et al., 2004); however, recent studies have shown that this may not be optimal since LL-37 upregulates many components of the inflammatory response (Braff et al., 2005) and may not act systemically since it is a peptide and therefore subject to proteases. Based on our current study, we propose that further investigations into the neutralization

of IL-4 and IL-13 in AD skin prior to VV vaccination could reduce the risk for AD patients to develop EV and allow these individuals to develop immunity against smallpox more safely.

#### Experimental Procedures

##### Patients

Subjects included 16 patients with AD (mean age: 31.8 years; 20%–60% skin involvement), 12 patients with psoriasis (mean age: 46.2 years; 15%–40% skin involvement), and 11 healthy individuals with no history of skin disease (mean age: 31.2 years). None of the patients had previously received oral corticosteroids or cyclosporin, and topical corticosteroids were not allowed for more than 1 week prior to enrollment. This study was approved by the institutional review board at National Jewish Medical and Research Center in Denver, and all patients gave written informed consent prior to participation in these studies.

##### Mice

BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Cnlp*<sup>-/-</sup> (CRAMP knockout) mice were obtained from R.L. Gallo (Veterans Affairs Medical Center, San Diego, CA) and were backcrossed onto the BALB/c background. TLR-3 knockout and STAT-6 knockout mice were purchased from the Jackson Laboratory. All animal protocols were approved by the Institutional Animal Care and Use Committee at National Jewish Medical and Research Center. This institution has an animal welfare assurance number (A3026-1) on file with the Office of Protection and Research Risks.

##### Virus Source and Culture

The Wyeth strain of VV was obtained from the Centers for Disease Control and Prevention (Atlanta, GA). VV was propagated by using HeLa S3 cells (ATCC#CCK-2.2) as previously described (Howell et al., 2004). Virus was harvested, yielding infectious virions in the form of intracellular mature virions (Schmelz et al., 1994).

##### Human Skin Explant Cultures

Punch biopsies (2 mm) were obtained from nonlesional skin of each donor and were placed in a 96-well plate and RPMI (Cellgro) supplemented with 10% FCS (Gemini Bio Products). Biopsies were cultured in the presence of media alone or  $2 \times 10^5$  pfu VV for 24 hr. After the exposure period, media were removed, and biopsies were submerged in 10% buffered formalin for immunohistochemical staining, or Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) for RNA isolation.

In some experiments, monoclonal anti-human IL-4 (1  $\mu$ g/ml; R&D Systems, Minneapolis, MN) and monoclonal anti-human IL-13 (1  $\mu$ g/ml; R&D Systems) were added to the skin biopsies for 24 hr prior to infection with VV. After the infection, biopsies were cultured for an additional 24 hr and prepared for RNA isolation.

To evaluate the antiviral activity of LL-37, skin biopsies were infected with  $2 \times 10^5$  pfu VV for 6 hr. After the incubation, VV was removed, and the biopsies were washed with media to remove the remaining VV. LL-37 (100  $\mu$ M) was added to the skin biopsies and allowed to incubate for an additional 18 hr. RNA was isolated for analysis of VV gene expression.

##### Murine Skin Explant Cultures

The dorsal thorax of all mice was clipped and treated with the depilatory agent Nair to remove hair. A total of 72 hr after hair removal, mice were euthanized via carbon dioxide asphyxiation. Punch biopsies (6 mm) were collected from the dorsal thorax and immediately placed in a 96-well plate and RPMI (Cellgro) supplemented with 10% FCS (Gemini Bio Products). Murine skin was cultured in the presence of media alone or  $2 \times 10^5$  pfu VV for 24 hr. After the exposure period, media were removed, and biopsies were submerged in Tri-Reagent (Molecular Research Center, Inc.) for RNA isolation.

STAT-6 knockout mice were first treated with murine IL-4 (50 ng/ml; R&D Systems) and IL-13 (50 ng/ml; R&D Systems) for 24 hr prior to infection with VV.

##### Keratinocyte Cell Culture

Normal human epidermal keratinocytes were cultured in serum-free Keratinocyte Growth Media (Clonetics, San Diego, CA) prepared from the essential nutrient solution Keratinocyte Basal Medium supplemented with 10 ng/ml epidermal growth factor, 0.4  $\mu$ g/ml hydrocortisone, bovine pituitary extract, and antibiotics. HaCaT cells, a human keratinocyte cell line, were cultured in Dulbecco's modified Eagle's media (Cellgro) supplemented with 10% FCS (Gemini Bio Products) and 1% of the following until confluent: penicillin/streptomycin, L-glutamine, minimal essential medium with nonessential amino acids (GIBCO-BRL), and MEM vitamins solution (GIBCO-BRL).

To investigate the effects of the Th2 cytokines, cells were cultured in the presence and absence of IL-4 (50 ng/ml; R&D Systems) and IL-13 (50 ng/ml; R&D Systems) for 24 hr prior to stimulation with VV. Cells were then infected with VV for an additional 24 hr. RNA was isolated for analysis of VV gene expression and LL-37 by real-time RT-PCR.

To evaluate the antiviral activity of LL-37, keratinocytes were infected with 0.05 pfu/cell VV for 6 hr. After incubation, VV was removed, and cells were washed with media to remove the remaining VV. LL-37 (0–100  $\mu$ M) was added to the cells and allowed to incubate for an additional 18 hr. RNA was isolated for analysis of VV gene expression.

To investigate the mechanism of LL-37 induction, Poly I:C (Amersham Biosciences, Piscataway, NJ) was added to keratinocytes for 24 hr. RNA was isolated for analysis of LL-37 gene expression by real-time RT-PCR.

##### Real-Time RT-PCR

Total RNA was isolated from skin biopsies by chloroform:phenol extraction and isopropanol precipitation according to manufacturer's guidelines (Molecular Research Center, Inc.). RNeasy Mini Kits (Qiagen, Valencia, CA) were used according to the manufacturer's protocol to isolate RNA from cell cultures and to further purify RNA from skin biopsies. RNA was reverse transcribed into cDNA and analyzed by real-time PCR by using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA) as previously described (Nomura et al., 2003). Primers and probes for human GAPDH, IFN- $\gamma$ , IFN- $\alpha$ 1, and IFN- $\beta$ 1 were purchased from Applied Biosystems. VV and LL-37 primer and probes were prepared as previously described (Ong et al., 2002; Howell et al., 2004). Primer and probe sequences used to assay CRAMP gene transcripts were: forward, 5'-GCTGATTCTTTTGACATCAGCTGTA-3', reverse, 5'-GCCAGCCGGAAATTTTCT-3'. Quantities of all targets in test samples were normalized to the corresponding GAPDH levels in cultured keratinocytes and skin biopsies.

##### VV Immunofluorescent Staining

Paraffin-embedded tissues were cut at 5  $\mu$ m on frosted microscope slides. Using toluene and a series of ethanol washes, slides were deparaffinized and then rehydrated. Skin sections were then blocked with 5% BSA in Super Block (ScyTek Laboratories, Logan, UT) containing 10% nonimmune donkey serum (Jackson Laboratories, West Grove, PA) for 60 min. Slides were then stained with a mouse anti-E3L antibody (gift from Dr. Bernard Moss, NIH/NIAD) directed against an early viral protein located in the nucleus and cytoplasm of infected cells (Amegadzie et al., 1991) or control mouse IgG1 at 4°C overnight. Slides were washed with PBS/Tween 0.05%, followed by incubation with a Cy3-conjugated donkey anti-mouse IgG (Jackson Laboratories). Immunohistochemical staining was visualized with confocal microscopy (Leica, Wetzlar, Germany). Slides were coded to ensure patient anonymity. Images were collected at 40 $\times$ , and levels of mean fluorescence intensity were measured with Slidebook 3.0 (Intelligent Imaging Innovations, Denver, CO). Mean fluorescence intensity (MFI) was determined for each exposure group and was reported as mean MFI  $\pm$  SE.

##### LL-37 Protein Expression

Paraffin-embedded tissues were cut into 5  $\mu$ m sections, deparaffinized, rehydrated, and then stained with rabbit anti-LL-37 (5  $\mu$ g/ml) as previously described (Ong et al., 2002). Slides were coded to ensure patient anonymity. Images were collected at 40 $\times$  magnification, and the intensity of the immunostaining was scored on

a scale from 0 to 3, with 0 indicating no staining and 3 indicating the most intense staining.

#### Statistical Analyses

All statistical analysis was conducted by using Graph Pad Prism, version 3.01 (San Diego, CA). Statistical differences in gene expression or protein staining between multiple groups was determined by using a one-way analysis of variance (ANOVA), and significant differences were determined by a Tukey-Kramer test (Tukey, 1977).

#### Supplemental Data

Supplemental Data including four figures and Experimental Procedures are available at <http://www.immunity.com/cgi/content/full/24/3/341/DC1/>.

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