Overexpression of IL-1α in Skin Differentially Modulates the Immune Response to Scarification with Vaccinia Virus

Tian Tian[1], Luzheng Liu[1], Eva-Jasmin Freyschmidt[2], George F. Murphy[3], Thomas S. Kupper[1] and Robert C. Fuhlbrigge[1,2]

Transepidermal inoculation of vaccinia virus (VV), or scarification, has been used effectively for the induction of specific and long-lasting immunity to smallpox and is superior to other routes of immunization. Scarification of individuals with atopic skin disease or immune deficiency, however, can lead to persistent viral replication and result in significant morbidity and mortality. These effects of scarification presumably reflect the unique immunological properties of skin and the immune cells resident in, or recruited to, the site of inoculation. To explore these phenomena, we utilized transgenic mice engineered to overexpress IL-1α, a critical mediator of cutaneous inflammation, in the epidermis. Following scarification with VV, both transgenic and wild-type mice develop local pox. At high doses of VV, IL-1α transgenic mice recruited immune cells to the inoculation site more rapidly and demonstrated enhanced T-cell and humoral immune responses. At limiting doses, however, IL-1α transgenic mice could effectively control virus replication without formation of pox lesions or activation of a memory response. This study suggests that IL-1 might be useful as an adjuvant to enhance antiviral immunity and promote safer vaccination strategies; however, understanding the balance of IL-1 effects on innate and adaptive immune functions will be critical to achieve optimal results.

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INTRODUCTION

Smallpox, caused by variola virus, can be prevented by immunization with vaccinia virus (VV). Although most immunizations are delivered by subcutaneous or intramuscular injection, effective smallpox vaccination requires inoculation of live VV into the skin—a process termed as scarification—and the production of an epidermal pox reaction. Although VV scarification in a normal host results in specific and long-lasting immunity, in atopic and immunodeficient patients, viral replication can outstrip immune control, leading to devastating morbidity and mortality (Bray, 2003). Previous studies have shown that the balance and interaction of innate and acquired immune defense mechanisms at the cutaneous interface are crucial elements that determine the speed and character of the immune response (Kupper and Fuhlbrigge, 2004). Based on these observations, we hypothesize that the outcome of scarification is dependent on the state of immune-response elements at the site of inoculation, and that manipulation of this environment can alter the immune response to viral challenge.

Interleukin-1 is a pleiotropic cytokine and a primary mediator of cutaneous inflammation, serving as an important link between innate and acquired immune responses (Dinarello, 1996; Murphy *et al.*, 2000). IL-1 is produced by many cells in response to infection or activation, and it has multiple effects, including endothelial activation, enhanced recruitment of leukocyte, increased production of both T helper 1 (Th1)- and T helper 2 (Th2)-related cytokines, and alterations of the hypothalamic thermoregulatory set point. Adjuvant effects of IL-1 include both increased antibody production and T-cell memory responses. In humans and in mice, IL-1 activity is mediated by two homologous cytokines, IL-1α and IL-1β, that bind the same receptor(s) and elicit essentially indistinguishable responses mediated by the signal adaptor protein MyD88. The agonist effects of IL-1α and IL-1β are balanced by coexpression in all tissues of IL-1 receptor antagonist. Skin antigen-presenting cells (APCs: monocytes, macrophages, Langerhans cells, and dendritic cells (DCs)) produce primarily IL-1β. Unstimulated keratinocytes, in contrast, contain large amounts of preformed and biologically active IL-1α, as well as significant quantities of immature IL-1β. Furthermore, keratinocytes also synthesize IL-1 receptor

[1]*Department of Dermatology, Brigham and Women's Hospital, Boston, Massachusetts, USA;* [2]*Division of Immunology, Children's Hospital, Boston, Massachusetts, USA and* [3]*Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts, USA*

Correspondence: Dr Robert Fuhlbrigge, Harvard Skin Disease Research Center, Brigham and Women's Hospital, 221 Longwood Avenue, Boston, Massachusetts 02115, USA. E-mail: rfuhlbrigge@partners.org

Abbreviations: AD, atopic dermatitis; APC, antigen-presenting cell; DC, dendritic cell; ELISPOT, enzyme-linked immune spot; GFP, green fluorescent protein; ILN, inguinal lymph node; K14, keratin 14; LN, lymph node; SFC, spot-forming cell; Th1, T helper type 1; Th2, T helper type 2; VV, vaccinia virus; WR, Western reserve; WT, wild-type

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antagonist and express the two forms of IL-1 receptors (agonist receptor (IL-1R1) and decoy receptor (IL-1 R2)). The epidermis, therefore, is unique in that all elements of the IL-1 axis are represented. Disruption of this complex regulatory network is associated with profound inflammatory disease (Dinarello, 1996).

Numerous published studies have explored the biological consequences of disrupting the natural balance of the IL-1 network in skin by creating transgenic mouse strains that overexpress IL-1 agonists or antagonists under control of the human keratin 14 (K14) promoter, which directs expression to the basal layer of the keratinized epidermis (Vassar *et al.*, 1989; Groves *et al.*, 1995, 1996; Rauschmayr *et al.*, 1997). In this study, we have used K14/IL-1α transgenic mice to examine the influence of increased epidermal IL-1α on the immune response to vaccinia skin scarification.

RESULTS

T-cell immune response is enhanced and skewed toward Th1 in K14/IL-1α mice scarified with VV

Keratin 14/IL-1α and wild-type (WT) mice were inoculated by scarification at the base of the tail with 2×10^6 PFU of Western reserve (WR)–green fluorescent protein (GFP) virus and monitored by daily inspection. The vaccination site became erythematous by day 3 and developed a pustule near the end of the first week in both WT and transgenic mice. During the second week, pustules progressed to scab lesions, which fell off in the third week, leaving a small scar. The visual appearance and time course of the pox lesions were similar in these two strains, as well as the C57BL/6 strain (Liu *et al.*, 2006), and closely resembles local reactions of humans after scarification. None of these mice showed secondary pox lesions. T cells harvested from inguinal lymph node (ILN) and spleen of K14/IL-1α and WT mice and stimulated with VV-infected APCs showed no induction of IFN-γ or IL-2 production at day 3 after scarification. By day 7, however, both IFN-γ and IL-2 production were increased, with a substantial enhancement noted in K14/IL-1α mice compared with WT control mice (Figure 1a). On average, ILN T cells from K14/IL-1α mice produced threefold more IL-2 (995.8 ± 40.4 *vs* 277.5 ± 4.3 pg ml^{-1}) and eightfold more IFN-γ (28816.7 ± 6840.5 vs 3273.3 ± 80.8 pg ml^{-1}) than WT mice on day 7 postscarification. Splenic T cells from K14/IL-1α mice also showed increased cytokine production, but the

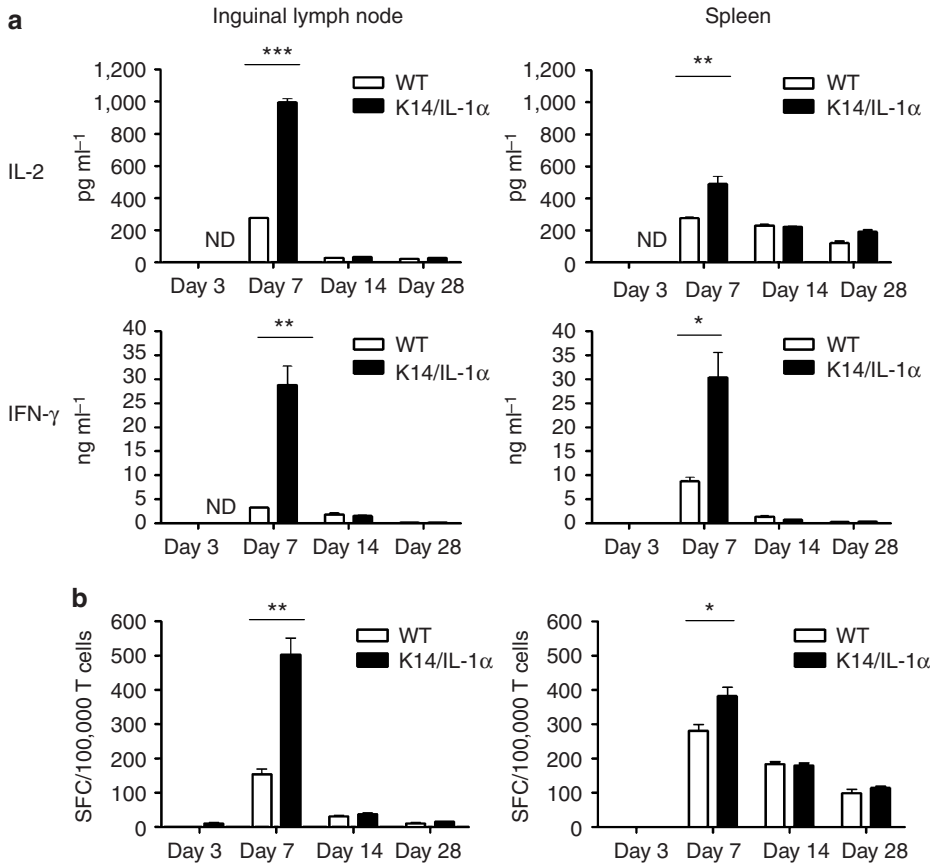

Figure 1. Keratin 14/IL-1α mice develop stronger Th1 immune response following vaccinia scarification compared with WT mice. K14/IL-1α and WT mice were skin-scarified at the base of their tails with 2×10^6 PFU of WR-GFP VV. At indicated time points after immunization, purified inguinal LN or splenic T cells were cultured *in vitro* with infected syngeneic (APC). (**a**) IL-2 and IFN-γ in the culture supernatant measured after 40 hours. Bars represent means ± SE and are representative of four independent experiments. (**b**) Frequency of vaccinia-specific T cells measured by IFN-γ ELISPOT assay. Data are expressed as spot-forming cells (SFC) per 10^6 T cells. Bars represent means ± SE and are representative of two independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$; ND, not detected.

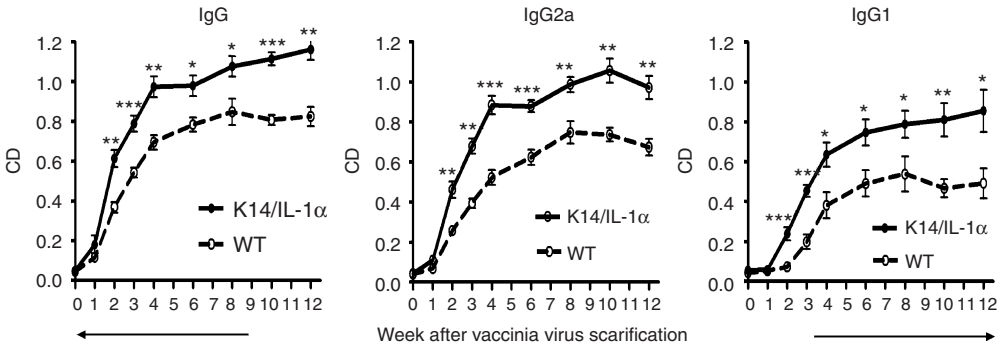

Figure 2. Kinetics of vaccinia-specific antibody response following scarification with VV. K14/IL-1α and WT mice were skin-scarified at the base of their tails with 2×10^6 PFU of WR-GFP VV. At various time points after immunization (weekly for weeks 1–4 and biweekly for weeks 6–12), mice from each group were bled and their sera were collected. Sera were also collected from control, uninfected (week 0) mice. The kinetics of the vaccinia-specific antibody response in the sera of individual mice at each time point was measured by ELISA. Data represent means ± SE using six mice per group and are representative of four independent experiments. $*P<0.05$, $**P<0.01$, $***P<0.001$.

differences were less dramatic. T-cell immune responses declined rapidly after day 7, with no significant difference in cytokine production between the two strains seen at 2 weeks after immunization. Four weeks after immunization, ILN T cells from both K14/IL-1α and WT mice stimulated with VV-infected APC produced only trace amounts of cytokines. Spleen T cells, in contrast, still generated significant amounts of IL-2 at day 14 and beyond, with no significant difference documented between transgenic and WT mice. No significant increase in IL-4 or IL-10 production was detected from ILN or splenic T cells from either strain in response to stimulation with VV-infected APCs (data not shown).

We further evaluated T-cell immunity by enumerating VV-reactive T cells following scarification using an IFN-γ enzyme-linked immune spot (ELISPOT) assay. T cells were isolated from spleen and ILN on days 3, 7, 14, and 28 after scarification, and the frequency of T cells responding to VV-infected APCs was measured by ELISPOT (Figure 1b). Consistent with the analysis of cytokine production by ELISA, the number of VV-specific IFN-γ-producing T cells 7 days after inoculation increased significantly in both the ILN and the spleen of K14/IL-1α mice relative to WT mice. In ILN, the number of VV-reactive T cells was greatly reduced at week 2 in both strains, and very few IFN-γ-producing T cells could be detected at 4 weeks after VV inoculation. Although the number of VV-reactive T cells in spleen also decreased at week 2, a significant number of IFN-γ-producing T cells were still present 4 weeks after inoculation in both K14/IL-1α and WT mice. To summarize, both K14/IL-1α and WT mice demonstrated a Th1-dominated immune response following VV scarification, with K14/IL-1α mice producing significantly greater numbers of IFN-γ-producing T cells and more Th1 cytokines than WT control mice in the first week after inoculation.

Production of antibody to VV is enhanced in K14/IL-1α mice

To assess the humoral immune response of K14/IL-1α and WT mice to VV, serum was collected weekly from day 0 to week 4 and then biweekly from week 6 to week 12 following scarification. Titer of VV-specific antibody in individual mice was determined by ELISA. All mice scarified with VV developed VV-specific IgG after 2 weeks. Antibody responses peaked at about week 6 and remained stable over the next 6 weeks. K14/IL-1α mice produced vaccinia-specific IgG earlier and at higher titer in comparison with WT mice. Measurement of the distribution of IgG subclasses was used as a surrogate for the Th1 *versus* Th2 bias of the immunological response. Although both vaccinia-specific IgG2a (Th1-associated) and IgG1 (Th2-associated) were produced after vaccinia scarification, the relatively stronger induction of IgG2a for all mice indicates a bias toward a Th1 cellular response as a result of VV inoculation (Figure 2).

K14/IL-1α mice recruit T cells and DCs to the site of inoculation more rapidly than WT mice

To assess lesion development and the kinetics of infiltration with T cells, we performed immunohistochemical staining of skin on days 0, 3, 7, and 10 following scarification with VV. Skin from unimmunized K14/IL-1α and WT mice (day 0) did not appear significantly different following hematoxylin-and-eosin or anti-CD3 staining at baseline (data not shown). Although pox lesions induced by VV scarification of WT and K14/IL-1α mice did not appear different by gross inspection (as noted above), there was a substantial difference in the histological appearance. At day 3 postscarification, scattered infiltrating cells were seen by hematoxylin-and-eosin stain in the dermis and hypodermis of WT mice (Figure 3). In contrast, a dense cellular infiltrate was observed in the skin of K14/IL-1α mice, particularly in the perifollicular regions. These infiltrates were composed primarily of mononuclear cells. Staining for CD3 showed very few T cells at the site of inoculation in WT mice, whereas strong CD3 + staining was evident at the site of inoculation in K14/IL-1α mice. Similarly, although major histocompatibility complex-II (MHC-II) + stained cells are seen in WT skin at day 3 postinnoculation, they are significantly increased (>4-fold) in the skin of K14/IL-1α mice. These cells are in the same perifollicular distribution as the CD3 + cells. Although some likely represent activated T cells, many are dendritic in shape and thus most likely are APCs. Although these findings hold for all

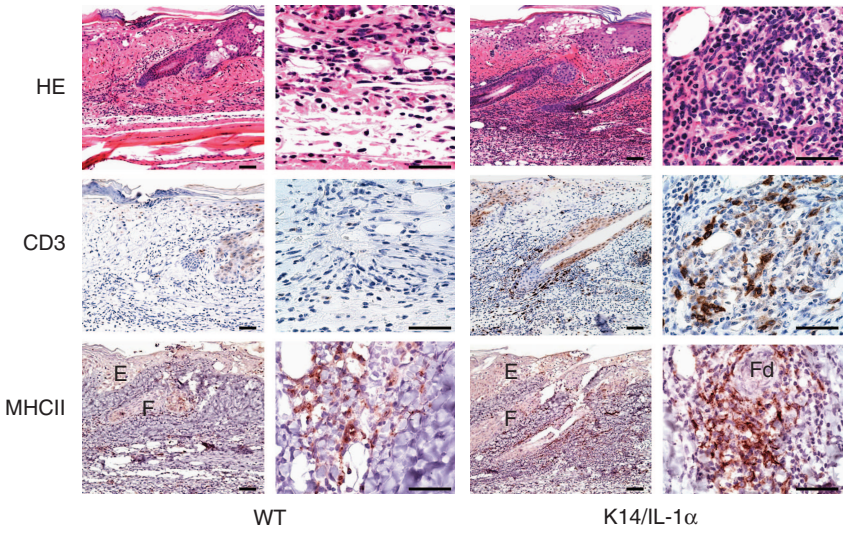

Figure 3. Histology of skin lesions from vaccinia-scarified WT and K14/IL-1α mice. Skin samples from the site of inoculation were obtained from WT and K14/IL-1α mice 3 days after scarification with 2×10^6 PFU of WR-GFP VV. Formalin-fixed paraffin-embedded tail-skin samples were stained with hematoxylin and eosin as well as anti-CD3 and anti-Ia (MHC-II) mAb. Labels indicate epidermis (E), proximal follicle (F), and distal follicle (Fd) regions. The data shown are representative of 3–4 individual mice per experimental group. Bar = 250 μm.

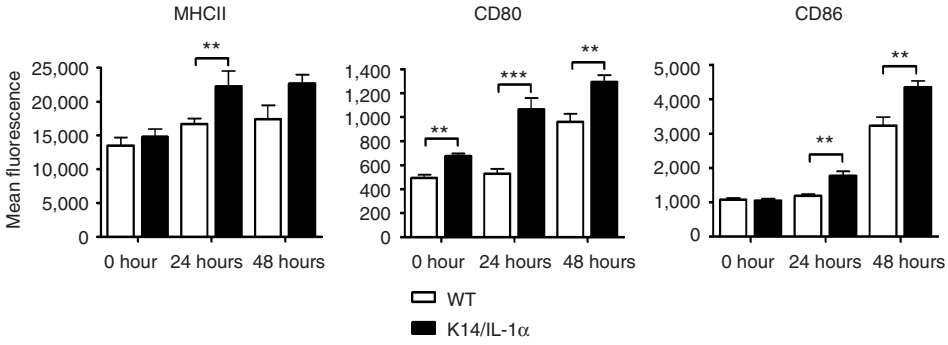

Figure 4. Phenotypic maturation of inguinal LN DC after vaccinia scarification. Cells recovered from ILNs harvested at the time points indicated were stained for CD11c and MHC-II, CD80, or CD86. Bars represent mean fluorescence intensity of MHC-II, CD80, and CD86 on CD11c+-gated cells. Data represent the means ± SE of 4–5 individual mice in one experiment. $*P<0.05$, $**P<0.01$, $***P<0.001$, n.s. = not significant.

time points examined (days 3, 7, and 10), they were first evident and most striking on day 3 (data for days 7 and 10 not shown). These combined data support the impression that there is a robust cutaneous immune response at the site of scarification with VV that consists primarily of T cells and MHC-II-positive APC, and that this response is augmented in K14/IL-1α mice compared with WT controls. Furthermore, these data highlight the lack of sensitivity of the gross appearance of lesions in assessment of the degree and character of the cutaneous immune response.

Phenotypic maturation of DCs in the draining lymph node is accelerated in K14/IL-1α mice after VV scarification

To explore the mechanism of enhanced immune response in K14/IL-1α mice, we examined DC from draining lymph nodes (LNs) for expression of surface markers related to DC activation and maturation. $CD11c^+$ cells from LNs of VV-naive K14/IL-1α mice expressed similar levels of MHC-II, CD86, and slightly higher levels of CD80 compared with WT mice, as assessed by flow cytometry (Figure 4). Scarification with VV resulted in increased expression of all three markers at 24 hours on $CD11c^+$ cells from K14/IL-1α, but not in WT mice. At 48 hours postinfection, $CD11c^+$ cells from WT mice began to show upregulation of these maturation markers, but still expressed significantly lower levels than K14/IL-1α mice. To assess APC functions directly, cell suspensions of spleen and ILN from VV-naive K14/IL-1α and WT mice were infected *in vitro* with VV and cultured for 40 hours with responder splenocytes from vaccinia-immune WT mice. Assay of culture supernatants for IFN-γ by ELISA showed no difference in APC function between spleen cells of K14/IL-1α and WT mice, whereas APCs from skin-draining LNs of K14/IL-1α mice supported increased IFN-γ production relative to WT LN cells (data not shown). These results support the

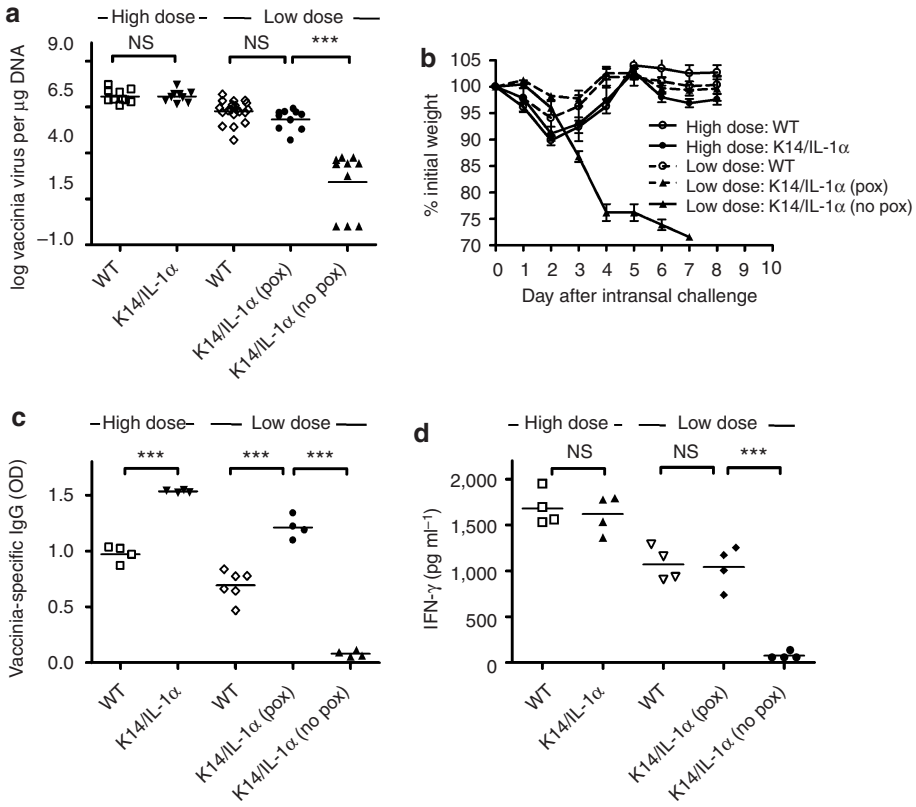

Figure 5. Influence of low-dose VV on protection in mouse challenge models. K14/IL-1α and WT mice were skin-scarified at the base of their tails with 2×10^6 PFU (high dose) or 5×10^3 PFU (low dose) of WR-GFP VV. All of the WT mice, immunized with either high dose ($n = 10$) or the low dose ($n = 20$) VV, developed pox lesions. Of K14/IL-1α mice immunized with low-dose virus ($n = 20$), half did not form pox lesions ($n = 10$). (**a**) Inoculated skin was removed at 7 days postimmunization and vaccinia viral DNA content was determined by quantitative PCR. This method can detect levels as low as 1 virus copy per µg DNA. Data shown represent the individual values (symbols), and means (bars) of 9–20 mice per group in two experiments. ***$P < 0.001$, n.s. = not significant. (**b**) Five weeks after vaccination, mice received an intranasal challenge with 1×10^7 PFU of VV WR and were weighed daily. The data plotted represent the means ± SE for each group. (**c**) Vaccinia-specific IgG in the sera of individual mice measured by ELISA 1 month after immunization. Data represent the means ± SE of 4–6 mice per group. ***$P < 0.001$. (**d**) Thirty-five days postinfection, whole splenocytes were prepared from each mouse and stimulated with *in vitro*-infected syngeneic APC. IFN-γ in the culture supernatant was measured after 40 hours. Bars represent means ± SE for mice in each group. ***$P < 0.001$; n.s., not significant.

hypothesis that excess IL-1α in the epidermis promotes the maturation of DC draining to the local LNs, which, in turn, enhances the T-cell immune response to vaccinia scarification. In summary, although the size, character, and duration of pox lesions developing in response to skin scarification appeared similar in WT and K14/IL-1α mice, functional and phenotypic analyses of the immune response show a substantial difference between these strains.

K14/IL-1α mice show a threshold adaptive immune response to scarification

Based on our observations that excess IL-1α in skin enhanced the immune response to VV inoculation, we hypothesized that this would support protection against a lethal challenge at lower, and presumably safer, doses of VV used for immunization. To explore this hypothesis, K14/IL-1α mice and WT mice immunized with high (2×10^6 PFU) or low (5×10^3 PFU) dose VV WR-GFP by skin scarification were assessed for resistance to lethal intranasal challenge at 30 days postimmunization. Somewhat surprisingly, whereas a viral dose of 5×10^3 PFU/mouse inoculated via scarification still resulted in pox lesions at the inoculation site in WT mice, only 50% of K14/IL-1α mice developed pox reactions at this dose. This observation held through multiple repetitions of the experiment with careful attention to preparation and dilution of the viral stock used. Quantitative PCR was used to assess viral load at the inoculation site. At day 7 postscarification, the number of VV copies present in the skin of K14/IL-1α mice that developed pox lesions following scarification with high or low doses of VV were comparable with that of WT control mice, whereas samples from K14/IL-1α mice that did not form pox lesions showed significantly diminished levels of VV DNA (Figure 5a).

This finding was important, as development of a pox lesion after vaccinia scarification has historically been accepted as evidence of successful immunization against variola. To assess the development of protective immune responses, mice inoculated by scarification with high- and low-dose vaccinia received an intranasal challenge of 10^7 PFU of WR strain VV, a dose that is lethal to nonimmune mice, 5 weeks

after initial immunization. Sham-immunized K14/IL-1α and WT mice lost weight rapidly after intranasal challenge and succumbed to infection or were euthanized (because of ⩾25% body weight loss) by 7 days postchallenge (data not shown). In contrast, K14/IL-1α and WT mice immunized with high-dose (2×10^6 PFU) VV WR-GFP showed only transient weight loss and all survived (Figure 5b). Low-dose immunization (5×10^3 PFU) also led to protective immunity in WT mice and in those K14/IL-1α mice that formed pox lesions, whereas the K14/IL-1α mice that did not develop pox lesions succumbed to infection with a time course similar to nonimmunized mice. Thus, development of a pox lesion at the site of inoculation appeared indicative of an immune response that resulted in protective immunity. To test this hypothesis, we measured VV-specific antibody production and T-cell IFN-γ production by WT and K14/IL-1α mice scarified with high- *versus* low-dose VV at 1 month postinoculation. Consistent with the responses seen with high-dose (2×10^6 PFU) inoculation, K14/IL-1α mice that developed cutaneous pox lesions when scarified with low-dose (5×10^3 PFU) VV had significantly higher vaccinia-specific antibody titers than did WT controls treated in the same fashion. However, K14/IL-1α mice that did not form pox lesions when scarified with low-dose (5×10^3 PFU) VV did not show detectable VV-specific antibody at 30 days postinoculation (Figure 5c). Similarly, production of IFN-γ by splenocytes stimulated with VV-infected APCs at 1 month postinoculation was comparable for K14/IL-1α mice that developed pox lesions and WT control mice following scarification with high or low doses of VV, although splenocytes of K14/IL-1α mice that did not form pox lesions did not produce significant IFN-γ in response to VV-infected APC (Figure 5d).

DISCUSSION

In humans, induction of protective immunity to smallpox by immunization with VV depends on the delivery of virus to the epidermis by scarification and is characterized by development of an epidermal ''pox'' reaction. Indeed, vaccination by intradermal, subcutaneous, and intramuscular strategies have all been shown to result in lower neutralizing antibody titers and a reduced vaccinia-specific cytotoxic T lymphocyte response than vaccination by scarification (McClain *et al.*, 1997). From early in the history of smallpox vaccination, it has been recognized that certain patient groups are at risk of complications of the scarification technique (Lofquist *et al.*, 2003). Vaccinia necrosum, or progressive vaccinia, is seen in severely immunocompromised patients and leads to severe morbidity and death from overwhelming infection. Eczema vaccinatum, which occurs in patients with active or quiescent atopic dermatitis (AD), reflects an inability of the atopic host to control the spread of virus from the inoculation site and can result in substantial tissue injury or death. Generalized vaccinia infection, which occurs in patients without AD, is not typically lethal but can also cause significant injury. Owing to concerns regarding these complications of vaccine use, and the apparent elimination of smallpox infection worldwide, widespread vaccination with VV was halted in the United States in 1972 and worldwide in the years to follow. Recent concerns about the possible reemergence of smallpox, or its deliberate release as a bioterror agent, has led to renewed interest in vaccination for the armed forces, frontline healthcare personnel, and perhapsthe general population. At the same time, a relative increase over the past 30 years in the percent of the world population with AD, and immune deficiency related to organ transplantation, chemotherapy treatments, and HIV disease has spurred interest in understanding the biology of vaccinia-immune responses and driven efforts to design safer vaccination protocols.

Although the risks of live viral vaccination in immune-deficient patients appear obvious, the basis for the severe adverse effects of vaccinia scarification seen in atopic patients has not been defined. Vaccination with a live pathogen inherently involves a ''race'' between growth and effective immune response. In the normal host, APCs carrying antigen migrate from the site of infection through afferent lymphatics to the draining LNs where they act to prime T cells. Antigen-specific CD4 + and CD8 + T cells activated in skin draining LNs become imprinted with a skin-homing phenotype and will home preferentially to the inoculation site (Liu *et al.*, 2006), where they can lyse infected target cells and promote resolution of the infection (Robert and Kupper, 1999). Complications of live pathogen immunization can result from an inability to respond to a specific infection or an imbalance that delays control of growth of the pathogen. Large DNA viruses, including poxviruses and herpesviruses, commonly encode homologs of cytokines, chemokines, and their receptors as a strategy to evade the host immune response (Alcami, 2003). Genes expressed by VV encode proteins that inhibit complement, bind IL-1 and IL-18, inhibit CC chemokines, and block IFN function, among others (Kotwal *et al.*, 1990; Alcami and Smith, 1992; Symons *et al.*, 1995; Alcami *et al.*, 1998; Smith *et al.*, 2000; Moss and Shisler, 2001). Together, these viral products alter the evolution of inflammatory signals and limit the recruitment of leukocytes to the site of infection, providing the virus with a crucial advantage in the race with the immune response.

In the case of vaccinia scarification, the balance and interaction of innate and acquired immune mechanisms in the skin are crucial. The dose of VV used for scarification has been empirically optimized such that the immune system of a normal host usually ''wins,'' leading to control of virus growth and long-term immunity. In AD patients, however, the immune response is altered or inhibited such that VV replicates faster than the immune system responds, leading to greater local tissue injury and a risk for dissemination.

Atopic skin, therefore, appears to represent a specialized environment that promotes VV growth and/or inhibits anti-viral immunity. Evidence for both functions has been described. Activation and expansion of cytotoxic anti-viral T cells, for example, are associated with Th1-type cytokines (for example,, IL-12 and IFN-γ). Atopic skin is characterized, however, by reduced expression of Th1 cytokines and increased expression of Th2 cytokines (for example, IL-4 and IL-13) (Hamid *et al.*, 1994). VV infection of human

keratinocytes has also been shown to induce expression of Th2-promoting cytokines and other immunoregulatory factors, including transforming growth factor beta, IL-10, and IL-13, suggesting that vaccinia may skew local cytokine production against the generation of a protective Th1 and cytotoxic T-cell responses (Liu *et al.*, 2005). Innate immune mechanisms are also altered in atopic hosts. LL-37, as an example, is a cathelicidin antimicrobial peptide produced by mammalian skin that exhibits antiviral activity against purified VV. Recent studies have demonstrated that atopic skin has decreased LL-37 expression and supports increased replication of VV compared with normal or psoriasis skin (Howell *et al.*, 2006). IL-4 and IL-13 treatment of vaccinia-infected keratinocytes, in turn, enhanced replication of VV, whereas it downregulates LL-37 expression. These and other alterations in innate and adaptive immune responses seen in the skin of atopic individuals may promote local growth of VV and restrict or delay the expansion or function of Th1 and Tc1 cells that form the basis of the antiviral response.

As noted previously, the balance of IL-1 agonists and antagonists plays a central role in the regulation of cutaneous immune responses (Groves *et al.*, 1995, 1996; Murphy *et al.*, 2000). It is then, perhaps, not surprising to find inhibitors of IL-1 function among the immune modulators produced by vaccinia. The VV gene *B15R*, for example, encodes a soluble IL-1 receptor, which binds soluble IL-1β and inhibits functional responses (Spriggs *et al.*, 1992). Interestingly, experimental deletion of B15R from VV accelerates the appearance of symptoms of illness and mortality in mice infected intranasally with this modified virus, indicating that some of the adverse effects of respiratory infection may be related to IL-1 levels in the lung (Alcami and Smith, 1992). In contrast, immunization with modified VV Ankara with the soluble IL-1β receptor gene deleted results in a better CD8+ T-cell memory response and confers higher levels of protection against subsequent lethal respiratory infection with WT VV (Staib *et al.*, 2005). Myxoma virus, a poxvirus that infects rabbits, encodes a protein named M13L-PYD, which blocks production of the IL-1 family cytokines by inhibiting caspase-1 activation in the cytoplasmic ''inflammasome,'' thus preventing the maturation of IL-1β from inactive precursor to active cytokine. Knockout viruses that do not express this protein are markedly attenuated, showing decreased viral dissemination and enhanced inflammatory responses at sites of infection (Johnston *et al.*, 2005). Similarly, the vaccinia viral gene *B13R* (SPI-2) encodes a serpin homolog that inhibits caspase-1. Although deletion of *B13R* diminishes virulence, this recombinant virus still elicited potent humoral, T-cell helper, and cytotoxic T-cell immune response in the mice (Legrand *et al.*, 2004). Predicting the effects of vaccinia infection on IL-1 production and function are even more complex in the context of skin disease. In patients with atopic skin disease (AD), the ratio of IL-1 receptor antagonist to IL-1α in stratum corneum samples from uninvolved skin of the face, trunk, and extremities is significantly increased due to a decrease in IL-1α and an increase in IL-1 receptor antagonist production (Terui *et al.*, 1998). IL-1β mRNA, in contrast, is increased at baseline in lesional skin from patients with AD and has been shown to rise in response to patch testing with house dust mite antigen (Junghans *et al.*, 1998; Jeong *et al.*, 2003). The relative impact of these competing effects on IL-1 function in the context of vaccinia scarification is, at present, unknown.

In this study, we used a K14/IL-1α transgenic mouse model to examine the role and potential benefit of cutaneous IL-1α as a modulator of immune response to VV inoculated by scarification. As shown, K14/IL-1α mice scarified with 2×10^6 PFU of vaccinia mounted an earlier and stronger Th1 immune response and an enhanced humoral immune response compared with WT control animals. These observations are consistent with previously reported effects of IL-1 on Th1 responses in a *Leishmania* infection model (Filippi *et al.*, 2003, Von Stebut *et al.*, 2003). K14/IL-1α mice also recruited T cells and APCs more rapidly to the site of inoculation and displayed more rapid maturation of DCs arriving at the draining LNs. The balance of forces affecting innate immune function was highlighted by results of our studies with reduced vaccinia inoculation doses. When the dose used for scarification was decreased to 5,000 PFU, WT mice still developed pox lesions and produced significant vaccinia-specific antibody and cell-mediated responses. K14/IL-1α transgenic mice, however, showed much lower levels of virus present in the site of inoculation at 7 days, and more than half did not develop a local pox reaction or show subsequent evidence of a memory immune response. We hypothesize that low-dose VV inoculated by scarification in WT mice is able to suppress the local innate immune response sufficiently to allow limited local growth of virus, leading ultimately to strong activation of the adaptive immune response and eventual control of the infection. In K14/IL-1α mice, in contrast, innate immune function is sufficiently enhanced to clear the virus in some mice before they can be growth sufficient to create a pox and/or activate an adaptive immune response. High-dose inoculation, in contrast, overwhelms the protective effect of excess IL-1 in the transgenic animals, resulting in pox lesion formation and strong immune responses in both WT and IL-1 transgenic mice.

In summary, the observation that epidermal IL-1α enhances T-cell and antibody responses to VV suggests that IL-1α could be used as an adjuvant in humans, increasing the effectiveness of VV inoculation in at-risk populations and potentially supporting the use of lower inoculation doses or attenuated virus strains. It is clear, however, that there is an optimal balance between innate and adaptive immune function that must be considered in any such strategy, and that development of a pox lesion may still be the most suitable indicator of successful, protective immunization.

MATERIALS AND METHODS

Virus

Recombinant thymidine kinase-negative (TK^-) VV NP-S-EGFP (WR-GFP, a kind gift of Dr Bernard Moss, NIH) and TK^+ VV WR strain were expanded in HeLa cells and titered in CV-1 cells (American Type Culture Collection, Manassas, VA) using standard procedures (Earl *et al.*, 1998).

Mice

Wild-type FVB/N mice were purchased from the Jackson Laboratories (Bar Harbor, ME). The K14/ IL-1α 1.2 line was generated in inbred FVB/N mice (Groves *et al.*, 1995). Heterozygous transgenic mice from the K14/IL-1α strain were bred to nontransgenic siblings or to FVB/N WT mice to produce synchronized delivery of sufficient numbers of animals for scarification experiments. F1 progeny were screened by PCR with primers for the transgenic K14 promoter. Mice lacking transgene were designated as WT littermate controls. As has been described previously, unstimulated transgenic K14/IL-1α mice display only subtle signs of skin inflammation, such as minor erythema around the snout and mild alopecia as they age, relative to WT mice. All mice were handled in accordance with guidelines set out by the Center for Animal Resources and Comparative Medicine at Harvard Medical School.

Immunizations and challenges

Mice were immunized with VV WR-GFP by scarification as described (Liu *et al.*, 2006). Briefly, mice were anesthetized with 2,2,2 tribromoethanol (250 mg kg^{-1}; Sigma, St Louis, MO) by intraperitoneal injection with a target of 25–30 minutes of immobility. Five microliters of trypsinized virus at varying titers were placed on the base of the tail. The inoculation site was scarified with a 28-g needle (500-μl insulin syringe) by poking 25 times and scratching 25 times, endeavoring to stay within the superficial epidermis and to minimize bleeding. For the high-dose/low-dose studies, a single aliquot of virus was prepared for use with all mice at each dose (WT and K14/IL-1α) to minimize the risk of errors in dilution and handling. For challenge experiments, mice were anesthetized with isoflurane and inoculated intranasally with 10^7 PFU of VV WR (10 μl of 5×10^8 PFU ml^{-1} stock was administered into each nostril). The individual mice were weighed and checked for survival daily (Snyder *et al.*, 2004). Mice that lost more than 25% of their body weight were euthanized.

In vitro restimulation assay

Single-cell suspensions were prepared from ILNs and spleens of immunized mice. Red blood cells were lysed with lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na EDTA, pH 7.3). T cells were purified from infected ILN or spleens using a Pan-T-cell isolation kit (Miltenyi Biotec, Auburn, CA). In all experiments using T cells, the purity of the cells was ⩾95% as determined by flow cytometry. Splenocytes from naive syngeneic mice were infected with VV WR-GFP at an multiplicity of infection of 20 for 8 hours and irradiated with 3,300 Rad for use as APCs. For stimulation assays, a total of 3×10^5 purified T cells or whole splenocytes were stimulated with 3×10^5 infected splenocytes in 96-well flat-bottomed plates. After 40 hours, cytokines in the culture were measured by ELISA (BD Pharmingen, San Jose, CA).

VV-specific IFN-γ ELISPOT

Interferon-γ ELISPOT assay was performed using the BD ELISPOT kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Briefly, ELISPOT plates were coated with an anti-IFN-γ mAb (capture mAb) overnight at 4 °C. The plates were washed and blocked with complete RPMI 1640 with 10% fetal calf serum. Purified T cells and VV-infected APCs prepared as in the *in vitro* restimulation assay were added to the wells and cultured at 37 °C for 40 hours. The plates were washed and a biotinylated anti-IFN-γ mAb was added for 2 hours, followed by washing. Streptavidin-alkaline phosphatase was added for 1 hour, followed by washing and development of a color reaction using the 3-amino-9-ethylcarbazole substrate reagent provided. The reaction was stopped by adding water, and an immunospot analyzer (Series 3A; Cellular Technology, Shaker Heights, OH) was used to count the spots (Tian *et al.*, 2005).

Measurement of VV-specific antibody

Vaccinia virus-specific antibody was determined at the indicated time points by ELISA. A 96-well EIA/RIA plate (cat. no. 3369, Costar, Lowell, MA) was coated with UV-inactivated VV suspension containing 10^5 PFU per well VV WR-GFP in PBS at 4 °C overnight. The plate was washed and blocked with PBS with 10% fetal calf serum and 0.05% Tween-20 (PBST) for 2 hours at room temperature. Serum samples were diluted at 1:200 with PBST and incubated at 4 °C for 1 hour, washed 3 times, and incubated for an additional 1 hour at room temperature with horseradish peroxidase conjugated goat anti-mouse IgG, IgG1, or IgG2a (Southern). The plate was again washed and developed using 3,3′, 5,5′ tetramethylbenzidine (BD Biosciences) as a substrate. Color development was stopped by adding 2% H_2SO_4 to each well. Relative ELISA units were calculated from the absorbance at 450 nm wavelength (OD450) on a Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA).

Quantitative PCR for determination of viral load

Vaccinia viral load was evaluated by quantitative real-time PCR as described (Freyschmidt *et al.*, 2007). Briefly, inoculated skin and various organs were harvested at defined time points after scarification, and DNA was purified with the DNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. PCR was performed with the Bio-Rad iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The primers and TaqMan probe used in the quantitative PCR assay are specific for the vaccinia ribonucleotide reductase Vvl4L. The sequences of the primers are as follows: forward, 5′-GACACTCTGGCAGCCGAAAT-3′; and reverse, 5′-CTGGCGGCTAGAATGGCATA-3′. The TaqMan probe was synthesized by Applied Biosystems (Foster City, CA) with 5′-labeled with FAM and 3′-labeled with TAMRA. The sequence of the probe is 5′-AGCAGCCACTTGTACTACACAACATCCGGA-3′. Amplification reactions were performed in a 96-well PCR plate (Bio-Rad) in a 20-μl volume containing 2 × TaqMan Master Mix (Applied Biosystems), 500 nM forward primer, 500 nM reverse primer, 150 nM probe, and the template DNA. Thermal cycling conditions were 50 °C for 2 minutes and 95 °C for 10 minutes for one cycle. Subsequently, 45 cycles of amplification were performed at 94 °C for 15 seconds and 60 °C for 1 minute. Viral load was determined by establishing a standard curve from DNA of a VV stock with a previously calculated PFU determined by plaque assay. Corresponding cycle threshold values obtained by the real-time PCR methods were plotted on the standard curve to estimate sample viral load.

Histological analysis

Inoculated skin was harvested from 3–4 mice per group at each time point. Skin was preserved in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, anti-CD3, or anti-Ia antibody. Images were obtained with a Nikon E600 microscope and a Nikon EDX-35 digital camera. The images were assembled in Adobe Photoshop 7.0.

Flow cytometric analysis

Inguinal lymph nodes were excised from immunized mice and a single-cell suspension prepared by digestion with 250 μg ml^{-1} Liberase CI (Roche Applied Science, Indianapolis, IN) in RPMI for 40 minutes at 37 °C. Cells were stained with APC-anti-CD11c and with PE-anti-CD80, PE-anti-CD86, or IA/IE (BD Pharmingen). The incidence of positive cells and geometric mean fluorescence intensity were determined by flow cytometry (BD FACSCanto, San Jose, CA) and analyzed using FACSAria software (BD Biosciences).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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