

Vaccinia Virus Induces Strong Immunoregulatory Cytokine Production in Healthy Human Epidermal Keratinocytes: a Novel Strategy for Immune Evasion

Luzheng Liu,¹ Zhan Xu,² Robert C. Fuhlbrigge,¹ Victor Peña-Cruz,³ Judy Lieberman,² and Thomas S. Kupper^{1*}

Harvard Skin Disease Research Center, Department of Dermatology, Brigham and Women's Hospital, Boston, Massachusetts¹; CBR Institute for Biomedical Research, Boston, Massachusetts²; and Department of Immunology and Infectious Disease, Harvard School of Public Health, Boston, Massachusetts³

Received 4 November 2004/Accepted 8 February 2005

Introgenic cutaneous infection with vaccinia virus (VV) and naturally occurring systemic infection with variola virus both lead to the characteristic skin “pox” lesions. Despite significant medical experience with both viruses, surprisingly little is understood about the interactions between these poxviruses and healthy resident skin cells. In recent years, it has become clear that skin plays an essential role in modulating both innate and adaptive immune responses, in part by producing and responding to a variety of cytokines and chemokines upon stimulation. Antagonists of many of these compounds are encoded in poxvirus genomes. Infection of skin cells with poxvirus may lead to a unique pattern of cytokine and chemokine production that might alter the cutaneous immune surveillance function. In this study, we infected primary cultures of human skin cells with VV and monitored antigen expression, virus replication, and cytokine production from the infected cells. While T cells, Langerhans cells, and dermal dendritic cells were infected abortively, keratinocytes, dermal fibroblasts, and dermal microvascular endothelial cells (HMVEC-d) all supported the complete virus life cycle. In contrast to the robust viral replication in fibroblasts and HMVEC-d, only limited viral replication was observed in keratinocytes. Importantly, VV infection of keratinocytes led to up-regulation of immunoregulatory and Th2 cytokines, including transforming growth factor β , interleukin-10 (IL-10), and IL-13. We propose that the rapid induction of keratinocyte Th2 and immunoregulatory cytokines represents a poxvirus strategy to evade immune surveillance, and the limited viral multiplication in keratinocytes may be a protective mechanism to help the immune system “win the race.”

In recent years, accumulating evidence has revealed that in addition to providing the physical barrier between the body and the environment, skin cells can actively modulate both innate and adaptive immune responses, in part by producing and responding to a variety of cytokines and chemokines upon stimulation. While the cytokine and chemokine production in skin, especially in keratinocytes, has been shown to be important in contact hypersensitivity responses, chronic skin inflammatory diseases, and cutaneous malignancies, little has been done to investigate the immunological response of resident skin cells upon viral infections (23, 37, 41, 45). Of particular interest to us is cutaneous infection by poxviruses. Poxviruses have been shown to enter their hosts either by the respiratory route or through the skin. Natural infection with many poxviruses leads to the characteristic skin “pox” lesions, and the severity of infection varies dramatically from one species to another, causing anywhere from a local, self-limiting infection to a devastating systemic disease, such as smallpox (40). In humans, inoculation of vaccinia virus (VV) through the epidermis leads to successful immunization against smallpox in

the majority of the vaccinees (44). However, serious adverse responses are not uncommon, especially in patients with pre-existing immune abnormalities (25). One complication, eczema vaccinatum, occurs primarily in patients with atopic dermatitis, a chronic inflammatory skin condition affecting 5 to 15% of the pediatric population and somewhat fewer adults. Atopic dermatitis is associated with a Th2-dominated dysregulation of cytokine production (1, 15). These observations suggest that the skin microenvironment is crucially involved in the development of protective immune responses as well as pathological reactions following poxvirus infection. Studying the interaction between VV and skin cells is therefore critical for the understanding of the immunogenicity as well as the pathogenesis of adverse responses following VV scarification. Knowledge gained from these studies will be valuable for the development of optimal poxvirus-based vaccines that are both safe and effective, even for patients with defective immunity.

Like other poxviruses, VV has been shown to utilize multiple immune evasion strategies to negatively modulate the host immune response (40). Multiple early VV gene products are directed at inhibiting the innate immune response (2–4). The adaptive immune response is also targeted. Th1 cytokines such as tumor necrosis factor alpha (TNF- α) and gamma interferon (IFN- γ) have been found to play an integral role in controlling vaccinia virus infections, by enhancing CD8⁺ T-cell-mediated cytotoxic function and directly inhibiting viral replication. Sol-

* Corresponding author. Mailing address: Harvard Skin Disease Research Center, Department of Dermatology, Brigham and Women's Hospital, 77 Avenue Louis Pasteur, Suite 672, Boston, MA 02115. Phone: (617) 525-5550. Fax: (617) 525-5571. E-mail: tkupper@partners.org.

uble antagonistic receptor homologues for TNF- α and IFN- γ are produced by VV-infected cells (36). Conversely, the Th2 cytokines seem to be permissive for poxvirus replication, as insertion of interleukin-4 (IL-4) in the mousepox genome rendered a genetically resistant strain of mice susceptible to the lethal viral challenge (19). It is unknown whether VV infection of target cells could alter the Th2 cytokine production or function. We hypothesize that infection of skin cells by VV may lead to a unique pattern of cytokine and chemokine production that could inhibit the virus-controlling Th1 response and favor the development of a Th2 response.

To explore these issues, we infected primary cultures of isolated human skin cells with VV, including keratinocytes, dermal fibroblasts, dermal microvascular endothelial cells (HMVEC-d), Langerhans cells (LC), dermal dendritic cells (DC), and skin-homing cutaneous leukocyte antigen (CLA)-positive T cells (16). Viral antigen expression and replication, as well as the cytokine production profile, were monitored in the infected cells. We show that VV infects skin cells of both hematopoietic origin and nonhematopoietic origin, but the infection is abortive in the former cell types and productive in the latter. To our surprise, VV replicates only at a low level in keratinocytes, in contrast to the robust viral replication in dermal fibroblasts and endothelial cells. Most importantly, while vaccinia virus infection of HMVEC-d led to a general "shutdown" of cytokine and chemokine expression, VV induced multiple immunoregulatory cytokines in the infected keratinocytes, including the negative immunoregulatory and Th2 cytokines transforming growth factor β (TGF- β), IL-13, and IL-10. These results suggest a novel immune evasion mechanism employed by poxviruses to skew local cytokine production against the generation of protective Th1 and cytotoxic T-lymphocyte (CTL) responses. In the case of VV infection, these strategies are insufficient to permit unrestrained viral growth in most immunocompetent individuals. However, in immunocompromised patients and those with a preexisting predilection towards generating Th2 responses, they may enable this usually innocuous virus to overwhelm immune defenses and cause serious diseases.

MATERIALS AND METHODS

Cells. Healthy human skin was obtained as discarded material from facelift surgery or reduction mammoplasty in accordance with the policies of the human research committee of Brigham and Women's Hospital, Boston, MA. Primary human keratinocyte cultures were generated as described previously, with slight modification as noted (8). Briefly, small skin pieces were incubated at 37°C for 1 h with 1.2 U/ml grade II dispase (Roche, Indianapolis, IN) in complete RPMI 1640 medium. Epidermis was gently teased away from dermis and incubated in 0.25% trypsin-0.1% EDTA for 5 min at 37°C to dissociate cells. Following centrifugation for 5 min at 300 \times g, the cells were resuspended and plated in Gibco keratinocyte-sfm culture medium (see below), and exponentially growing cultures at approximately 30% confluency were used for serial passage. Keratinocyte differentiation was induced by culturing the cells in the presence of 1.5 mM calcium. Dermal fibroblasts were isolated from dermis by collagenase (90 U/ml) digestion and cultured in DF12 medium (see below). HMVEC-d cells (CC-2543; Cambrex BioScience, Walkersville, MD) were expanded from cryopreserved cells in EGM-2-MV medium (CC-3202; Cambrex BioScience) according to the vendor's instructions. All cultures were maintained at 5% CO₂ and 37°C. The cells were plated in six-well culture plates 1 or 2 days prior to infection.

Immature LC were isolated from healthy skin by discontinuous density gradient as described elsewhere (35). Mature LC and dermal DC were obtained by cell migration from epidermal and dermal sheets, respectively, as previously described (34). The purity of the cell populations was generally >90%, as de-

termined by flow cytometry (data not shown). The immature LC were CD1a^{hi}, CD83⁻, and HLA-DR^{int}, the mature LC were CD83⁺ and HLA-DR^{hi}, and the dermal DC were CD1a^{int}, CD83⁺, and HLA-DR^{hi} (data not shown). The isolated cells were purified in complete RPMI 1640 and used within 12 h. Total CD3⁺ T cells were purified from healthy human peripheral blood mononuclear cells by using a pan T-cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Cell purity was typically >90% as determined by flow cytometry (data not shown). CLA⁺ T cells were purified by magnetic bead separation from the above CD3⁺ T cells using rat anti-human CLA monoclonal antibody (MAb) HECA 452 (BD Pharmingen, San Diego, CA) followed by mouse anti-rat kappa microbeads (Miltenyi Biotec). Antibodies not provided in isolation kits were purchased from BD Pharmingen. The purity of CLA⁺ T cells was typically over 85% by flow cytometry analysis. The African green monkey kidney fibroblast cell line, CV-1, was a kind gift from Judy Lieberman (Center for Blood Research, Boston, MA) and was maintained in complete Dulbecco's modified Eagle's medium (DMEM).

Cell culture medium. Complete RPMI 1640 medium is RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 2 mM L-glutamine, 100 μ g/ml streptomycin, and 0.5 mg/ml amphotericin. Gibco keratinocyte-sfm culture medium was supplemented with 25 μ g/ml bovine pituitary extract and 0.2 ng/ml epidermal growth factor (EGF; Invitrogen, Grand Island, NY) plus 0.3 mM CaCl₂ and antibiotics (39). DF12 medium is a 1:1 mixture of DMEM and F-12 medium (Mediatech, Herndon, VA) supplemented with 15% FCS, 10 ng/ml EGF, and antibiotics. Complete DMEM is DMEM supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics.

Virus stock and infection. Wild-type Western Reserve strain VV (WR-VV) was a kind gift from N. Manjunath (Center for Blood Research, Boston, MA). The viral stock was expanded and the titers were determined on CV-1 cells by standard procedures (13). Cells were cultured in six-well plates to reach ~85 to 95% confluence and infected with WR-VV at the indicated multiplicity of infection (MOI) in 0.5 ml/well of culture medium for 1 h. The cultures were then brought to 2 ml of medium/well and incubated for additional time periods as indicated before harvesting.

Intracellular staining for viral gene expression and flow cytometry. Mouse MAb TW2.3 and rat MAb19C2, recognizing early viral antigen (Ag) E3L and late viral Ag B5R, respectively (38, 47) (kind gifts from Bernard Moss, National Institutes of Health), were used to detect vaccinia virus gene expression in the infected cells. Cells were permeabilized and fixed with CytoPerm/CytoFix (BD Pharmingen) and incubated with the above Abs for 30 min on ice, followed by labeling with fluorescein isothiocyanate-conjugated goat-anti-mouse immunoglobulin polyclonal Ab (BD Pharmingen) and phycoerythrin-conjugated goat F(ab')₂ anti-rat immunoglobulin G (Southern Biotechnology Associates, Inc., Birmingham, AL). Samples were then processed using a FACSCalibur flow cytometer (Becton Dickinson Labware), and the data were analyzed using Flowjo software (Tree Star, Inc., Ashland, OR).

Plaque assay to determine viral replication. Cells were cultured in six-well plates to reach 85 to 95% confluence. Two extra wells of each cell culture were used to obtain cell number per well. The cultures were then infected with WR-VV at an MOI of 0.1 or 10 for 60 h. Cells were harvested and suspended in 200 μ l 10 mM Tris buffer (pH 8) before lysis by three freezing (dry ice-ethanol) and thawing (37°C water bath) cycles. The titer of vaccinia PFU per sample was determined by standard plaque assay on CV-1 monolayers (13). The PFU per cell was calculated as PFU per sample per number of cells at the beginning of the infection.

MicroSpot ELISA array to measure cytokine production from infected cell cultures. Cells were infected with WR-VV at an MOI of 10 for 24 h, and supernatant samples were collected and stored at -80°C until analysis. Uninfected culture supernatant samples were collected as controls. The concentrations of 16 cytokines in the supernatant samples were measured simultaneously with the MicroSpot enzyme-linked immunosorbent assay (ELISA) array technology at the Schleicher & Schuell Protein Array Facility (Schleicher & Schuell, Keene, NH) (21, 42). All the samples were processed simultaneously in triplicate. Briefly, capture MABs for human IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p40, IL-12 p70, IL-13, IFN-inducible protein 10 (IP10), soluble intercellular adhesion molecule 1 (sICAM-1), TNF receptor II (TNFR II), TNF- α , IFN- γ , vascular endothelial growth factor (VEGF), and TGF- β were arrayed on the three-dimensional nitrocellulose surface of the FAST slide. Supernatant samples and serial dilutions of cytokine standard cocktail were added (70 μ l/pad) onto the slide, which was incubated for 3 h on an orbital shaker at room temperature followed by three washes. The biotinylated detection antibody cocktail was then added (70 μ l/pad) onto the FAST slide. Following 1 h of incubation on the orbital shaker and three washes, 0.125 μ g/ml reporter molecule streptavidin-Cy5

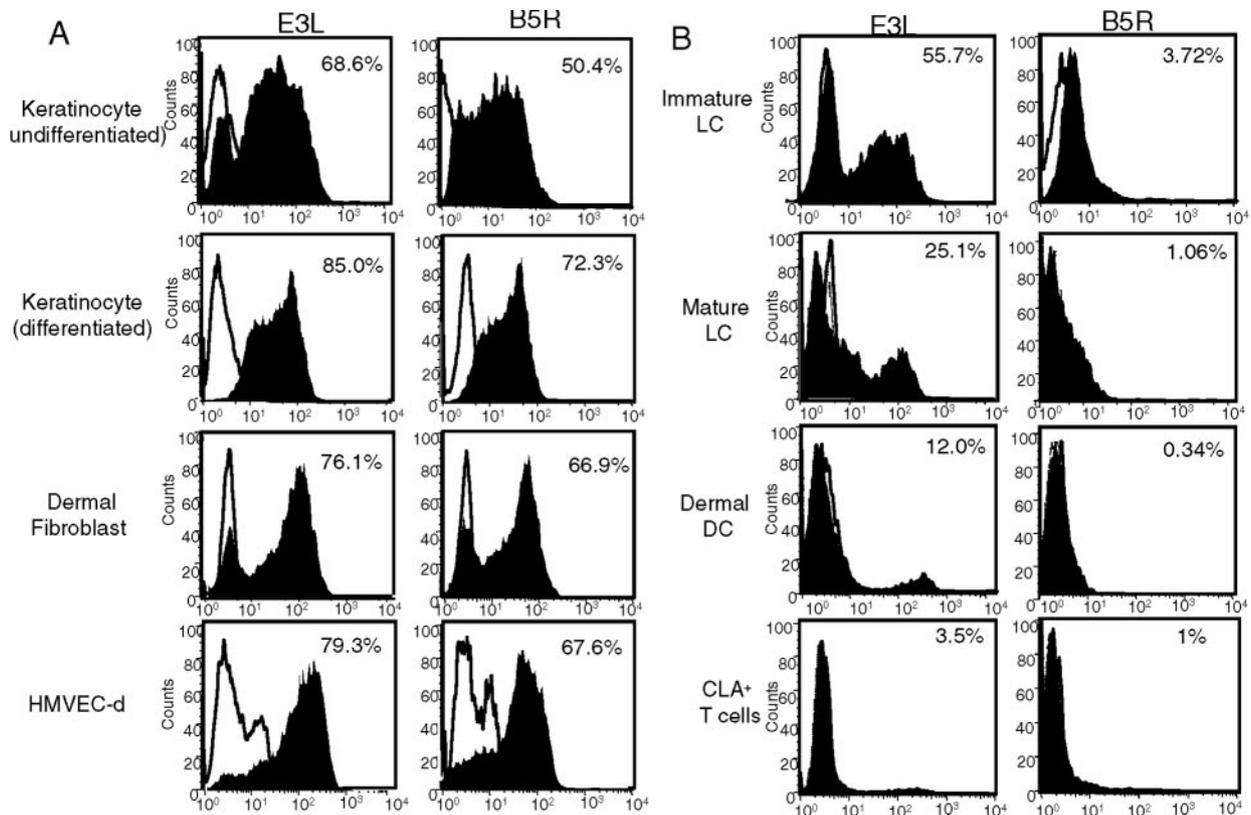


FIG. 1. Vaccinia virus infects various human skin cellular components in two distinct fashions. Primary cultures of human skin components were prepared and infected with WR-VV at an MOI of 10. Following 20 h of incubation, the expression of both early (E3L) and late (B5R) viral Ags was assessed with flow cytometry (filled histograms) as described in Materials and Methods. Uninfected cells were included as controls (unfilled histograms). The numbers are the percentages of viral Ag-positive cells in viable cell populations based on forward scatter and side scatter parameters. This is a representative result of three independent experiments.

was added and the slide was incubated in the dark for 45 min. After three washes, the slide was quickly rinsed, air dried, and scanned by GenePix scanners (Axon Instrument, Inc., Union City, CA). Data were analyzed using ArrayVision FAST software (Schleicher & Schuell, Keene, NH). The detection threshold for each cytokine is determined by the sensitivity of each antibody pair and has been assessed and provided by the manufacturer. The approximate sensitivity for IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-12 p70, IL-10, IL-13, TNFR II, TNF- α , IFN- γ , and TGF- β is \sim 3 to 15 pg/ml, 30 pg/ml for IP10, sICAM, and VEGF, and 100 pg/ml for IL-12 p40. Changes in cytokine concentrations of over 10-fold are considered significant. As pipetting and repetitive washes during the ELISA create recognizable errors between samples, we chose not to focus on any differences of less than 10-fold.

ELISA. Keratinocytes were infected with WR-VV at an MOI of 10 or irradiated with UVB at 100 μ J/cm² using a Stratalinker 2400 UV cross-linker with bulbs of peak emission at 312 nm (Stratagene, La Jolla, CA). After culturing for 24 h, supernatant samples were collected and stored at -80° C until analysis. Supernatants from untreated cells were collected as controls. The concentrations of TNF- α , IFN- γ , IL-12 p70, IL-13, IL-10, and TGF- β were measured using the Quantikine ELISA kits (R&D System, Minneapolis, MN) following the manufacturer's instructions. The approximate sensitivity for IL-13 is 32 pg/ml, and the sensitivity for other cytokines is \sim 5 to 10 pg/ml.

RESULTS

VV infects skin cells of different lineages in distinct fashions. To study VV infection of human skin cells, primary cultures of various human skin components, including epidermal keratinocytes (undifferentiated and differentiated), dermal fibroblasts, HMVEC-d, LC (immature and mature), dermal DC,

and CLA⁺ skin-homing T cells, were generated and infected with WR-VV in vitro at an MOI of 10. Infection was monitored by the expression of the early and late viral gene products E3L and B5R, respectively, using intracellular staining with anti-E3L and anti-B5R Abs. Among the cell types tested, two distinct patterns of VV infection were observed (Fig. 1A). High levels of both early (E3L) and late (B5R) viral gene expression were detected in keratinocytes, dermal fibroblasts, and HMVEC-d, suggesting productive infection in these cell cultures. The high percentages of viral Ag⁺ cells in these cultures indicate these cells are all highly susceptible to vaccinia virus infection. In contrast, early but not late viral gene expression was detected in cells of hematopoietic lineage, including LC, dermal DC, and CLA⁺ T cells (Fig. 1B), indicating abortive infection in these cell types. Except for immature LC, only a small percentage of the hematopoietic cells expressed early viral antigens despite the high infection dose used for this study. This could be due to a low-level virus entry into the cells or to intracellular mechanisms that suppress early viral gene expression. The fluorescence intensities of E3L staining in the limited number of cells supporting early gene expression did not differ significantly from those in the permissive cells (Fig. 1), suggesting that insufficient viral entry into the professional antigen-presenting cells (APCs) and T cells is a limiting factor.

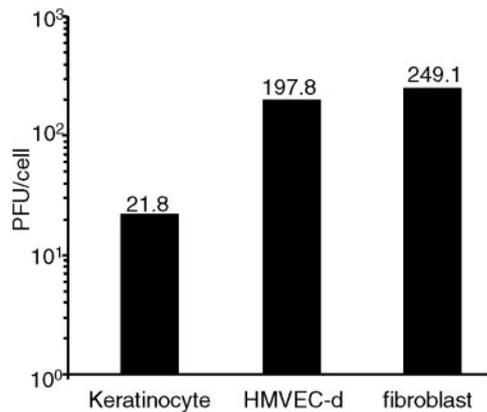


FIG. 2. VV undergoes only limited replication in keratinocytes, while replicating robustly in dermal fibroblasts and endothelial cells. Primary cultures of the indicated skin cells were infected with WR-VV at an MOI of 0.1 (0.1 PFU per cell). Sixty hours later, the numbers of virus particles in the cultures were determined by plaque assay and are shown as PFU per input cell. This is a representative result of three independent experiments.

Dermal fibroblasts and HMVEC-d but not keratinocytes support high levels of vaccinia virus replication. Replication of the VV genome occurs after the transcription and translation of early genes, but before that of the intermediate and late genes (31). Thus, the expression of the late viral Ag B5R indicates viral genome replication and potential viral propagation inside the infected cells. To quantitatively analyze viral replication in permissive skin cell cultures, known numbers of keratinocytes, dermal fibroblasts, and HMVEC-d were infected with WR-VV at a low MOI of 0.1 (0.1 PFU per cell). Sixty hours later, the total numbers of virus particles in the cultures were measured by plaque assay and the PFU per input cell was calculated as PFU per sample per number of cells at the beginning of the infection. Cell proliferation during the 60 h was negligible, since the cultures were 85 to 95% confluent at the time of the infection. As shown in Fig. 2, infectious virus particles were produced in all three types of skin cells, but to a different degree in each cell type. While the virus titers in HMVEC-d and dermal fibroblast cultures increased around 2,000-fold (197.8 and 249.1 PFU per cell, respectively) over the 60 h of infection, infection of keratinocytes only yielded 21.8 PFU virus particles per cell (1/10 of the viral yields from HMVEC-d and dermal fibroblasts). The robust vaccinia virus replication in HMVEC-d and dermal fibroblasts is typical for cell lines permissive for exponential viral multiplication. However, the low level of viral replication in keratinocytes was unexpected, since both early and late viral antigens were expressed in keratinocytes at levels comparable to, if not higher than, those seen in dermal fibroblasts and HMVEC-d (Fig. 1A). We have not observed semipermissive infection by VV in any other cell cultures tested. This phenomenon was not due to the early death of the infected keratinocytes, as the cytopathic effect of the virus on keratinocytes was much less severe than on fibroblasts at the end of the 60-h infection (data not shown).

We chose a low virus infection dose (MOI of 0.1) in the above experiment so that the vast majority of the viruses detected 60 h later were newly produced from the cultures instead of the input viruses. It is possible that the low virus yield

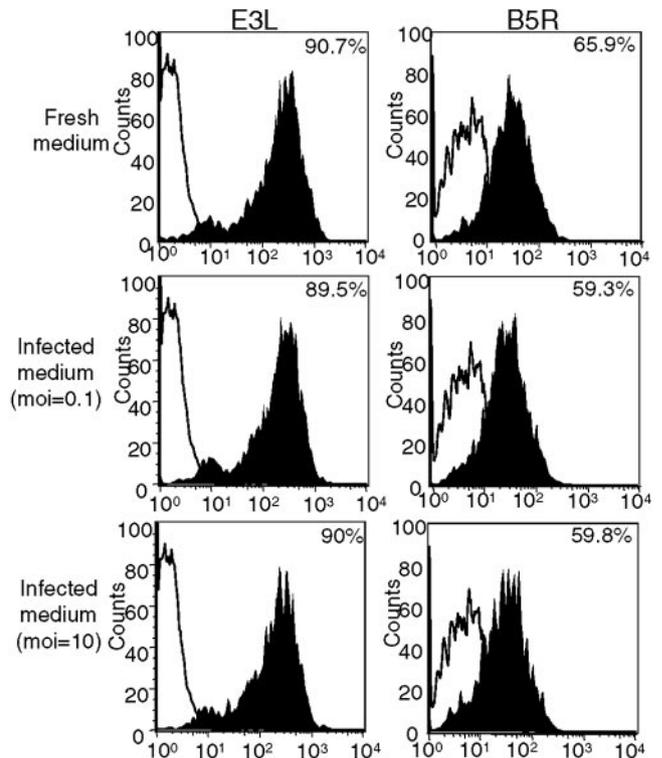


FIG. 3. VV-infected keratinocytes do not secrete soluble factors that can inhibit secondary viral infection. Keratinocytes were infected with WR-VV at an MOI of 0.1 (low dose) or 10 (high dose) for 24 h. Supernatant samples were harvested and stored at -80°C . Separate keratinocyte cultures were then infected with WR-VV at an MOI of 10 in the presence of either fresh medium or supernatant from low-dose- or high-dose-infected keratinocytes. Viral infection was monitored by the expression of early (E3L) and late (B5R) vaccinia virus antigens using flow cytometry, as described in the legend for Fig. 1. Unfilled histograms represent uninfected keratinocyte controls.

is due to soluble factors produced from the infected keratinocytes that can inhibit secondary infection in the culture or, alternatively, poor cell-to-cell transmission of VV in keratinocyte culture. To investigate these possibilities, we infected keratinocytes with WR-VV in either fresh keratinocyte medium or conditioned medium from WR-VV-infected keratinocyte cultures. Early and late viral gene expression was detected 20 h postinfection. As shown in Fig. 3, comparable levels of E3L and B5R were detected in keratinocytes infected in fresh medium and conditioned medium from low-dose or high-dose VV-infected keratinocytes, arguing against the inhibition of secondary infection by keratinocyte soluble factors. We then compared the viral propagation in different cell cultures at a higher virus-to-cell ratio to minimize the need for virus transmission between target cells. Known numbers of keratinocytes, dermal fibroblasts, and HMVEC-d were infected with WR-VV at an MOI of 10 (10 PFU per cell). Sixty hours later, the viral titers were measured by plaque assay and the PFU per input cell was calculated as described before. Increasing the initial viral input did not improve viral yields in all three cell cultures tested (Fig. 4 and 2). This result is expected for HMVEC-d and dermal fibroblasts, as viral yield reaches the plateau level (200 to 300 PFU per cell) over a 60-h incubation regardless of the

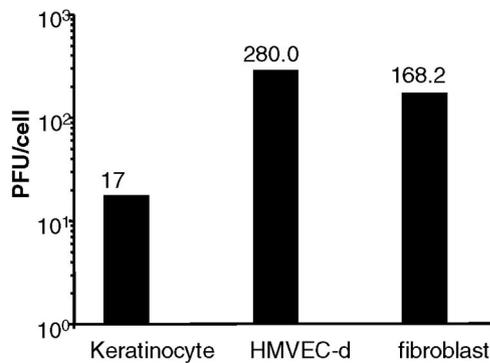


FIG. 4. The limited VV replication in keratinocytes is not due to poor cell-to-cell transmission. Known numbers of the indicated skin cells were infected with WR-VV at an MOI of 10 (10 PFU/cell). Sixty hours later, the numbers of virus particles in the cultures were determined by plaque assay and are shown as PFU per input cell. The numbers are the averages of duplicate samples.

input viral dose. In contrast, a 100-fold increase in the infection dose failed to improve the low viral yield (~20 PFU/cell) in keratinocytes, suggesting that the semipermissive fashion of vaccinia virus infection in keratinocytes is not due to poor virus spreading, but rather reflects intracellular mechanisms to inhibit late-phase viral production.

Keratinocytes and HMVEC-d show distinct patterns of cytokine production following vaccinia virus infection. The limited viral replication in keratinocytes suggests that biological functions of keratinocytes, such as cytokine production, may be largely preserved in the presence of the ongoing vaccinia virus infection. To further investigate the functional responses of skin cells following VV infection, supernatant samples from keratinocyte and HMVEC-d cultures were harvested at 24 h postinfection (MOI of 10) and subjected to quantitative analysis for a panel of cytokines and inflammatory mediators. We focused on keratinocytes and endothelial cells, because these two types of cells interact closely with both APCs and T cells in situ and have been implicated in the deviation of cutaneous immune responses toward a Th1 or Th2 phenotype in skin diseases such as psoriasis and atopic dermatitis (37). Sixteen molecules were measured simultaneously using an automated protein array system, and distinct patterns of cytokine production were observed in keratinocytes and HMVEC-d (Fig. 5).

In HMVEC-d, vaccinia virus infection led to greatly reduced production of IL-10, IL-13, TNFR II, and sICAM. With the exception of VEGF, the expression of other cytokines tested remained at low or undetectable levels (Fig. 5 and Table 1). This result was not surprising, as the virus is known to rapidly take over the cellular protein synthesis machinery and switch off the expression of cellular genes in permissive cells. However, in striking contrast, VV infection of keratinocytes resulted in significantly increased (at least 10-fold) levels of TGF- β , IL-10, IL-13, sICAM, and soluble TNFR, with the strongest up-regulation seen in TGF- β (from <15 pg/ml to 3.3 ng/ml). VEGF was the only cytokine tested that was detected at a higher level in HMVEC-d culture supernatant and at a lower level in keratinocyte supernatant. On the other hand, we did not observe any significant changes in levels of IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IFN- γ , IP10, and TNF- α following

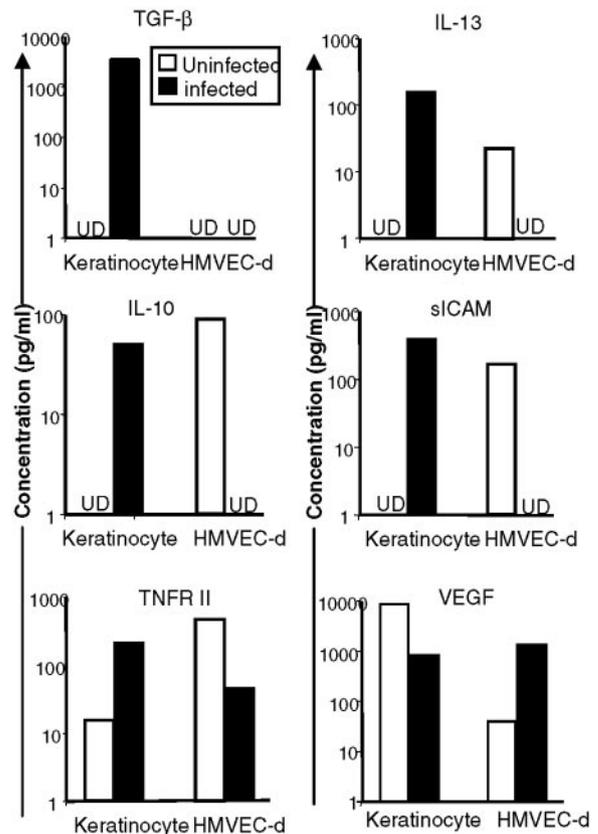


FIG. 5. Keratinocytes and HMVEC-d have distinct patterns of cytokine production following vaccinia virus infection. Keratinocytes and HMVEC-d were infected with WR-VV at an MOI of 10 and cultured for 20 h. The supernatant samples were collected and analyzed for a panel of cytokines and inflammatory mediators with the MicroSpot ELISA array system at the Schleicher & Schuell Protein Array Facility. Samples from uninfected cultures were included for comparison. Results represent the average cytokine concentrations detected from triplicate wells. UD, undetectable.

vaccinia virus infection of either keratinocytes or HMVEC-d (Table 1). Keratinocytes have been shown to respond to a variety of stimuli by producing cytokines and chemokines. To further investigate whether the Th2-prone profile of cytokine production from keratinocytes is a common response to general stimuli, or a specific response to vaccinia virus infection, we compared the cytokine production profiles of keratinocytes following VV infection or UVB irradiation. Six different Th1 or Th2 cytokines were measured by ELISA. Consistent with the results shown in Table 1, up-regulation of Th2 cytokines TGF- β , IL-10, and IL-13 was observed in vaccinia virus-infected keratinocytes, while the Th1 cytokines were undetectable in the supernatant. In contrast, a significant amount of the Th1 cytokine TNF- α was detected in UVB-irradiated keratinocyte culture supernatant (Table 2). TGF- β was also up-regulated in keratinocytes following UVB irradiation, but at a much lower level than after vaccinia virus infection. Taken together, these results demonstrate that VV infection of keratinocytes leads to up-regulation of TGF- β and Th2 cytokines.

TABLE 1. Cytokine concentrations in supernatants of VV-infected or uninfected keratinocytes and HMVEC-d cultures^b

Cytokine	Concn in supernatant (pg/ml)			
	Keratinocytes		HMVEC-d	
	Infected	Uninfected	Infected	Uninfected
IL-1 β	5.9	5.6	3.8	3.7
IL-2	7.8	<3 ^a	<3	<3
IL-4	<3	<3	<3	<3
IL-5	6.5	<5	<5	<5
IL-6	41.4	<5	12.5	47.9
IL-8	121.1	50.4	744.7	1,676.9
IL-12 p40	225.8	<100	<100	<100
IL-12 p70	29.2	22.7	21.6	27
IFN- γ	<3	<3	<3	<3
IP10	<30	64.3	<30	<30
TNF- α	17.5	5.4	10.5	15.5

^a Less than the detection threshold for the given cytokine.

^b Keratinocytes and HMVEC-d were either untreated or infected with VV at an MOI of 10 and cultured at 37°C for 24 h. Cytokine concentrations (pg/ml) in the supernatant samples were measured by MicroSpot ELISA array as described in Materials and Methods.

DISCUSSION

This is the first comprehensive study of vaccinia virus infection in healthy human skin cells. Two distinct patterns of infection were observed in different types of skin cells. Skin cells of hematopoietic lineage, such as LC and dermal DC, are relatively resistant to VV infection, and those that were infected did not support VV late gene expression or viral replication. This is consistent with a previous report from Bhardwaj's group that showed VV abortively infects human monocyte-derived DC (14). The resistance and abortive infection may allow the infected skin APCs to survive long enough to execute their functions—that is, to carry antigens to draining lymph nodes, or to activate skin-infiltrating lymphocytes *in situ*, and induce protective T-cell immune responses. This notion is supported by the observation that VV-infected mature monocyte-derived DC cultures induce strong CTL activity (7, 14). In contrast, VV readily establishes productive infection in epidermal keratinocytes, dermal fibroblasts, and HMVEC-d. Interestingly, despite the comparable levels of viral antigen expression among the permissive cell cultures, keratinocytes did not support a high level of VV replication as seen in dermal fibroblasts and HMVEC-d (Fig. 2). It is not clear at present how keratinocytes suppress vaccinia virus replication. Low-level virus production in keratinocytes is not due to inhibitory soluble factors from the infected cells (Fig. 3), nor inhibition of cell-to-cell spread (Fig. 4), suggesting intracellular pathways exist to limit VV replication at a late stage. It is possible that semi-permissive infection of keratinocytes may serve as a protective mechanism to prevent rapid virus production and dissemination in the skin surface layers following vaccinia virus inoculation.

In addition to providing a primary physical barrier between the body and the environment, skin cells also function in initiating innate inflammatory responses and shaping adaptive immune responses by producing and responding to inflammatory and immunoregulatory mediators in response to environmental insults (23, 37, 41, 45). We compared the expression levels of 16 cytokines and inflammatory mediators in kerati-

TABLE 2. Keratinocytes produced different cytokines responding to VV infection versus UVB irradiation

Cytokine	Concn (pg/ml) ^a		
	Untreated	UVB	VV infected
TNF- α	5.4	78.6	12.5
IFN- γ	6.33	7.29	9.2
IL-12 p70	20.1	15.8	24.6
IL-13	UD ^b	UD	103.7
IL-10	UD	UD	29.6
TGF- β	UD	164.8	2627

^a Keratinocytes were either untreated, irradiated with UVB at 100 μ J/s/cm², or infected with VV at an MOI of 10 and cultured at 37°C for 24 h. Cytokine concentrations (pg/ml) in the supernatant samples were measured by ELISA.

^b UD, undetectable by ELISA.

nocytes and HMVEC-d before and after VV infection. Our data show that while release of these factors is globally down-regulated in the infected endothelial cells, there is a remarkable up-regulation of multiple immunoregulatory and Th2-promoting cytokines, including TGF- β , IL-10, and IL-13, in the infected keratinocytes. Each of these cytokines was up-regulated by more than a factor of 10 and TGF- β by much more.

TGF- β is in general considered to be an inhibitory cytokine for the development of inflammation and Th1 responses. While TGF- β attracts neutrophils and lymphocytes to the injury site at early stages of inflammation, it inhibits their proliferation and function at later stages (29). The negative modulating role of TGF- β in Th1-dominated diseases has been well documented in autoimmune disease models using TGF- β -deficient animals (26). In addition, studies of lung epithelium from asthmatic patients suggest a role for TGF- β 1 in the pathogenesis of this characteristically Th2-dominated disease (43, 46). Recently, TGF- β has also been shown to be important in both the generation and the action of regulatory CD4⁺ and CD8⁺ T cells (18, 32). IL-10 is one of the best-studied suppressor cytokines for both innate and adaptive immune functions. It potently inhibits Th1 cells as well as the production of proinflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF- α (30). IL-10 produced from VV-infected keratinocytes may serve to prevent the induction of the proinflammatory cytokines. Indeed, we did not detect significant production of either Th1 or proinflammatory cytokines from vaccinia virus-infected keratinocytes (Table 1). Finally, IL-13 is also involved in the development of the Th2 response, with functions paralleling those of IL-4 (11, 28). These Th2 cytokines have been implicated in the pathogenesis of atopic dermatitis (1, 9, 24). We also observed significant up-regulation of sICAM and TNFR from the vaccinia virus-infected keratinocytes. Blocking T-cell LFA-1 locally by sICAM and binding of TNF- α by soluble TNFR may also contribute to local immunosuppression and are likely to enhance the inhibitory effects of the negative regulatory cytokines, such as IL-10, TGF- β , and IL-13 (5, 6, 17).

We did not observe any significant changes in levels of IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IFN- γ , IP10, and TNF- α following vaccinia virus infection of either keratinocytes or HMVEC-d (Table 1). However, these data need to be interpreted with caution, since a number of poxvirus early genes encode antagonistic cytokine receptor homologues that can

specifically bind to several regulatory cytokines, including IL-1 β , IFN- α , - β , - γ , and TNF- α (3, 4, 12, 22). These viral gene products may block the detection by MABs used in our detection assay. In unpublished studies, we have shown that coin-cubation of supernatant from VV-infected DC cultures with TNF- α -containing medium completely abrogated the ELISA detection of TNF- α (unpublished data). Rapid production of cytokine receptor homologues following the infection of target cells may provide the virus an opportunity to evade host innate as well as adaptive immune responses in the early stages following viral inoculation. Alternatively, the immunoregulatory and Th2 cytokines and chemokines from the infected keratinocytes may directly inhibit the production of some proinflammatory and Th1-inducing cytokines in the same cells.

We compared cytokine production from UVB-irradiated or VV-infected keratinocytes (Table 2). TNF- α , instead of IL-13 and IL-10, was produced from UVB-irradiated cells, and the induction of TGF- β was much weaker in the irradiated cells than in VV-infected cells. In addition, the IL-1 and IL-8 family of chemokines has been shown to be up-regulated following UV irradiation of keratinocytes (10, 27), and we failed to detect significant increases of these cytokines in vaccinia virus-infected keratinocyte culture supernatants. These results suggest that the up-regulation of the Th2 cytokines is not a response of keratinocytes to stimulation in general, but rather a specific response of these cells to VV infection. This notion is supported by studies of cytokine production from keratinocytes infected by other skin-tropic viruses. Increased Th1 cytokine IFN- γ , TNF- α , and IL-6 expression levels were observed in keratinocytes following varicella-zoster virus infection (33). On the other hand, human papilloma virus 2/27/57 infection led to an induction of IL-6, IL-10, and IP10 and an up-regulation of TGF- β (20).

While in many patients inoculation with smallpox vaccine induces local pain and swelling and systemic symptoms associated with viremia, the vast majority of vaccinees generate potent and long-lasting protective immunity against variola virus without any negative clinical consequence. It is reasonable to suspect that the strategies employed by vaccinia virus to evade the immune response, both previously described (40) and newly reported here, allow local replication of the virus such that a pox reaction and viremia can occur. However, they are insufficient to cause significant morbidity and mortality in patients with intact immune systems. Currently, it is still unclear how VV rapidly induces strong immune responses while utilizing multiple elegant strategies to evade the immune system. It will be important to investigate whether the intact physical barrier provided by the semipermissive keratinocytes is essential for preventing undesirable rapid virus dissemination and whether the presence of potent immune effector cells in vivo may overcome or reverse the pattern of the immunomodulating molecule production in skin following vaccinia virus infection.

Taken together, this is the first study to show that VV induces immunoregulatory and Th2-polarizing cytokines from infected keratinocytes. This may represent a novel VV strategy to alter the host immune response in favor of its own survival, albeit local and temporary in the majority of patients. It is currently under investigation whether the soluble factors produced from vaccinia virus-infected keratinocytes could in turn

modulate the maturation, differentiation, and functions of DC and T cells. It has been shown previously that VV counteracts the host immune response by blocking dendritic cell maturation, encoding soluble antagonistic receptor homologues for proinflammatory mediators and Th1 cytokines, and producing a soluble inhibitor that sequesters CC chemokines (2–4, 14, 36). Our study suggests that the induction of immunoregulatory cytokines by infected keratinocytes may work in concert with these immune evasion strategies to create a local cutaneous microenvironment that would antagonize virus-controlling Th1 and CTL responses and favor local viral replication.

In immunocompetent individuals, the host immune system can usually overcome these immune evasion mechanisms and completely clear the infection even after it has been present for several days. However, in patients with compromised cutaneous immune surveillance and impaired ability to regulate the Th1/Th2 balance (e.g., patients with atopic dermatitis), these immune evasion strategies may permit unrestrained viral growth and dissemination, resulting in the clinical condition known as eczema vaccinatum. Engineering recombinant VV strains that block immunoregulatory or Th2-polarizing cytokines may represent a new strategy for developing a safe smallpox vaccine.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant 1U19AI57330 to T. S. Kupper and by a research grant from the Dermatology Foundation to L. Liu.

We thank Elof Eriksson (Department of Plastic Surgery, Brigham and Women's Hospital, Boston, MA) for providing skin tissues, Jo-Ellen Murphy for preparing primary keratinocyte and fibroblast cell cultures, and Jay Omobono for help in editing the manuscript.

REFERENCES

1. Akdis, C. A., M. Akdis, A. Trautmann, and K. Blaser. 2000. Immune regulation in atopic dermatitis. *Curr. Opin. Immunol.* **12**:641–646.
2. Alcamí, A., and G. L. Smith. 1992. A soluble receptor for interleukin-1 beta encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. *Cell* **71**:153–167.
3. Alcamí, A., J. A. Symons, P. D. Collins, T. J. Williams, and G. L. Smith. 1998. Blockade of chemokine activity by a soluble chemokine binding protein from vaccinia virus. *J. Immunol.* **160**:624–633.
4. Alcamí, A., J. A. Symons, and G. L. Smith. 2000. The vaccinia virus soluble alpha/beta interferon (IFN) receptor binds to the cell surface and protects cells from the antiviral effects of IFN. *J. Virol.* **74**:11230–11239.
5. Anderson, M. E., and T. J. Siahaan. 2003. Targeting ICAM-1/LFA-1 interaction for controlling autoimmune diseases: designing peptide and small molecule inhibitors. *Peptides* **24**:487–501.
6. Baker, D., D. Butler, B. J. Scallon, J. K. O'Neill, J. L. Turk, and M. Feldmann. 1994. Control of established experimental allergic encephalomyelitis by inhibition of tumor necrosis factor (TNF) activity within the central nervous system using monoclonal antibodies and TNF receptor-immunoglobulin fusion proteins. *Eur. J. Immunol.* **24**:2040–2048.
7. Bonini, C., S. P. Lee, S. R. Riddell, and P. D. Greenberg. 2001. Targeting antigen in mature dendritic cells for simultaneous stimulation of CD4⁺ and CD8⁺ T cells. *J. Immunol.* **166**:5250–5257.
8. Boyce, S. T., and R. G. Ham. 1983. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.* **81**:33s–40s.
9. Breman, J. G., and D. A. Henderson. 2002. Diagnosis and management of smallpox. *N. Engl. J. Med.* **346**:1300–1308.
10. Caricchio, R., L. McPhie, and P. L. Cohen. 2003. Ultraviolet B radiation-induced cell death: critical role of ultraviolet dose in inflammation and lupus autoantigen redistribution. *J. Immunol.* **171**:5778–5786.
11. Deckers, J. G., S. De Haij, F. J. van der Woude, S. W. van der Kooij, M. R. Daha, and C. van Kooten. 1998. IL-4 and IL-13 augment cytokine- and CD40-induced RANTES production by human renal tubular epithelial cells in vitro. *J. Am. Soc. Nephrol.* **9**:1187–1193.
12. Dunlop, L. R., K. A. Oehlberg, J. J. Reid, D. Avci, and A. M. Rosengard. 2003. Variola virus immune evasion proteins. *Microbes Infect.* **5**:1049–1056.
13. Earl, P. L., N. Cooper, L. S. Wyatt, B. Moss, and M. W. Carroll. 1998.

- Preparation of cell cultures and vaccinia virus stocks, p. 16.16.4–16.16.6. *In* K. Struhl (ed.), *Current protocols in molecular biology*, 2nd ed., vol. 2. John Wiley & Sons, Inc., Mississauga, Ontario, Canada.
14. Engelmayer, J., M. Larsson, M. Subklewe, A. Chahroudi, W. I. Cox, R. M. Steinman, and N. Bhardwaj. 1999. Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. *J. Immunol.* **163**:6762–6768.
 15. Engler, R. J., J. Kenner, and D. Y. Leung. 2002. Smallpox vaccination: risk considerations for patients with atopic dermatitis. *J. Allergy Clin. Immunol.* **110**:357–365.
 16. Fuhlbrigge, R. C., J. D. Kieffer, D. Armerding, and T. S. Kupper. 1997. Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells. *Nature* **389**:978–981.
 17. Haak-Frendscho, M., S. A. Marsters, J. Mordenti, S. Brady, N. A. Gillett, S. A. Chen, and A. Ashkenazi. 1994. Inhibition of TNF by a TNF receptor immunoadhesin. Comparison to an anti-TNF monoclonal antibody. *J. Immunol.* **152**:1347–1353.
 18. Horwitz, D. A., S. G. Zheng, and J. D. Gray. 2003. The role of the combination of IL-2 and TGF- β or IL-10 in the generation and function of CD4⁺ CD25⁺ and CD8⁺ regulatory T cell subsets. *J. Leukoc. Biol.* **74**:471–478.
 19. Jackson, R. J., A. J. Ramsay, C. D. Christensen, S. Beaton, D. F. Hall, and I. A. Ramshaw. 2001. Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox. *J. Virol.* **75**:1205–1210.
 20. Jacobs, S., E. I. Grussendorf-Conen, I. Rosener, and A. Rubben. 2004. Molecular analysis of the effect of topical imiquimod treatment of HPV 2/27/57-induced common warts. *Skin Pharmacol. Physiol.* **17**:258–266.
 21. Knight, P. R., A. Sreekumar, J. Siddiqui, B. Laxman, S. Copeland, A. Chinnaiyan, and D. G. Remick. 2004. Development of a sensitive microarray immunoassay and comparison with standard enzyme-linked immunoassay for cytokine analysis. *Shock* **21**:26–30.
 22. Kotwal, G. J., S. N. Isaacs, R. McKenzie, M. M. Frank, and B. Moss. 1990. Inhibition of the complement cascade by the major secretory protein of vaccinia virus. *Science* **250**:827–830.
 23. Kupper, T. S., and R. C. Fuhlbrigge. 2004. Immune surveillance in the skin: mechanisms and clinical consequences. *Nat. Rev. Immunol.* **4**:211–222.
 24. Lane, J. M. 1969. Vaccination and Henoch-Schoenlein purpura. *N. Engl. J. Med.* **280**:781.
 25. Lane, J. M., and J. D. Millar. 1971. Risks of smallpox vaccination complications in the United States. *Am. J. Epidemiol.* **93**:238–240.
 26. Letterio, J. J., and A. B. Roberts. 1998. Regulation of immune responses by TGF- β . *Annu. Rev. Immunol.* **16**:137–161.
 27. Li, D., T. G. Turi, A. Schuck, I. M. Freedberg, G. Khitrov, and M. Blumenberg. 2001. Rays and arrays: the transcriptional program in the response of human epidermal keratinocytes to UVB illumination. *FASEB J.* **15**:2533–2535.
 28. Lucey, D. R., M. Clerici, and G. M. Shearer. 1996. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. *Clin. Microbiol. Rev.* **9**:532–562.
 29. Luethviksson, B. R., and B. Gunnlaugsdottir. 2003. Transforming growth factor- β as a regulator of site-specific T-cell inflammatory response. *Scand. J. Immunol.* **58**:129–138.
 30. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* **19**:683–765.
 31. Moss, B. 2001. Poxviridae: the viruses and their replication, p. 2849–2883. *In* P. M. Howley et al. (ed.), *Fields virology*, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
 32. K. Nakamura, A. Kitani, I. Fuss, A. Pedersen, N. Harada, H. Nawata, and W. Strober. 2004. TGF- β 1 plays an important role in the mechanism of CD4⁺ CD25⁺ regulatory T cell activity in both humans and mice. *J. Immunol.* **172**:834–842.
 33. Nikkels, A. F., C. Sadzot-Delvaux, and G. E. Pierard. 2004. Absence of intercellular adhesion molecule 1 expression in varicella zoster virus-infected keratinocytes during herpes zoster: another immune evasion strategy? *Am. J. Dermatopathol.* **26**:27–32.
 34. Pena-Cruz, V., S. Ito, C. C. Dascher, M. B. Brenner, and M. Sugita. 2003. Epidermal Langerhans cells efficiently mediate CD1a-dependent presentation of microbial lipid antigens to T cells. *J. Investig. Dermatol.* **121**:517–521.
 35. Pena-Cruz, V., S. Ito, M. Oukka, K. Yoneda, C. C. Dascher, F. Von Lichtenberg, and M. Sugita. 2001. Extraction of human Langerhans cells: a method for isolation of epidermis-resident dendritic cells. *J. Immunol. Methods* **255**:83–91.
 36. Reading, P. C., J. A. Symons, and G. L. Smith. 2003. A soluble chemokine-binding protein from vaccinia virus reduces virus virulence and the inflammatory response to infection. *J. Immunol.* **170**:1435–1442.
 37. Robert, C., and T. S. Kupper. 1999. Inflammatory skin diseases, T cells, and immune surveillance. *N. Engl. J. Med.* **341**:1817–1828.
 38. Schmelz, M., B. Sodeik, M. Ericsson, E. J. Wolffe, H. Shida, G. Hiller, and G. Griffiths. 1994. Assembly of vaccinia virus: the second wrapping cisterna is derived from the *trans* Golgi network. *J. Virol.* **68**:130–147.
 39. Schon, M., and J. G. Rheinwald. 1996. A limited role for retinoic acid and retinoic acid receptors RAR alpha and RAR beta in regulating keratin 19 expression and keratinization in oral and epidermal keratinocytes. *J. Investig. Dermatol.* **107**:428–438.
 40. Seet, B. T., J. B. Johnston, C. R. Brunetti, J. W. Barrett, H. Everett, C. Cameron, J. Sypula, S. H. Nazarian, A. Lucas, and G. McFadden. 2003. Poxviruses and immune evasion. *Annu. Rev. Immunol.* **21**:377–423.
 41. Soumelis, V., P. A. Reche, H. Kanzler, W. Yuan, G. Edward, B. Homey, M. Gilliet, S. Ho, S. Antonenko, A. Lauerma, K. Smith, D. Gorman, S. Zurawski, J. Abrams, S. Menon, T. McClanahan, R. de Waal-Malefyt, F. Bazan, R. A. Kastelein, and Y. J. Liu. 2002. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat. Immunol.* **3**:673–680.
 42. Stillman, B. A., and J. L. Tonkinson. 2000. FAST slides: a novel surface for microarrays. *BioTechniques* **29**:630–635.
 43. Wenzel, S. E., J. B. Trudeau, S. Barnes, X. Zhou, M. Cundall, J. Y. Westcott, K. McCord, and H. W. Chu. 2002. TGF- β and IL-13 synergistically increase eotaxin-1 production in human airway fibroblasts. *J. Immunol.* **169**:4613–4619.
 44. Wilson, C. B., and E. K. Marcuse. 2001. Vaccine safety-vaccine benefits: science and the public's perception. *Nat. Rev. Immunol.* **1**:160–165.
 45. Wolk, K., S. Kunz, E. Witte, M. Friedrich, K. Asadullah, and R. Sabat. 2004. IL-22 increases the innate immunity of tissues. *Immunity* **21**:241–254.
 46. Yamamoto, H., H. Teramoto, K. Uetani, K. Igawa, and E. Shimizu. 2002. Cyclic stretch upregulates interleukin-8 and transforming growth factor- β 1 production through a protein kinase C-dependent pathway in alveolar epithelial cells. *Respirology* **7**:103–109.
 47. Yuwen, H., J. H. Cox, J. W. Yewdell, J. R. Bennink, and B. Moss. 1993. Nuclear localization of a double-stranded RNA-binding protein encoded by the vaccinia virus E3L gene. *Virology* **195**:732–744.