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Identification of an Important Immunological Difference between Virulent Varicella-Zoster Virus and Its Avirulent Vaccine: Viral Disruption of Dendritic Cell Instruction

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Virulent varicella-zoster virus (VZV), a member of the human herpesvirus family, infects the majority of the human population. Similar to most other members of the human herpesvirus family, VZV has a narrow host range and does not infect mice or cells of murine origin (1). In humans, VZV is transmitted by aerosols and initially infects the upper respiratory tract. During the incubation period, VZV spreads to regional lymph nodes to infect T cells that subsequently transport virions to cutaneous sites (2). There, it efficiently replicates in epithelial cells and fibroblasts, causing the typical varicella rash. After primary infection, VZV establishes latency within sensory ganglia. From there, it can reactivate to cause herpes zoster many years later. This disease is characterized by papulovesicular skin lesions, mostly restricted to a single dermatome, and is frequently followed by severe pain. Cellular and humoral immune responses are essential for the control of VZV replication (3). VZV is the first human herpesvirus for which a live attenuated vaccine (vaccine Oka [V-Oka]) has been generated and approved (4). It is used successfully to immunize millions of children annually in the United States, Europe, and Japan and is now considered a prophylactic option in older individuals to prevent herpes zoster (5). Despite its unquestionable medical importance, the immunological difference between the vaccine and wild type virulent VZV has not been defined.

Virulent VZV efficiently spreads in human skin, whereas avirulent V-Oka only rarely causes clinical reactions. Thus, similar to other members of the human herpesvirus family, virulent VZV has evolved intricate immune-evasion strategies (6). These capabilities have been lost during the attenuation process generating the avirulent VZV vaccine. It should be possible to define virulence-associated evasion mechanisms by comparatively studying the capacity of virulent and avirulent VZV to subvert immune reactions. Dendritic cells (DCs) represent a prime target for immunoevasive viruses, because they couple innate to adaptive immunity to initiate efficient antiviral T cell responses. In the steady-state, two major DC subsets can be found in the skin (7): epidermal Langerhans cells (LCs) and dermal DCs. They form a network of sentinels that sense invading pathogens. Intriguingly, another DC subset derived from immigrating monocytes accumulates under inflammatory conditions. These inflammatory DCs replace steady-state DCs that disappear as
a result of migration to the lymph nodes or apoptosis (8, 9). Virulent VZV is known to infect monocyte-derived DCs (MDDCs) generated in vitro (10–12). However, no functional defect of immature MDDCs after VZV infection has been defined (10, 11).

Powerful Th1-like adaptive immune responses are essential to achieve virus control. To promote Th1 responses, DCs must provide three signals: signal 1 is delivered by Ag presentation through specialized surface molecules; signal 2 is mediated by costimulatory molecules on the surface, such as CD40, CD80, and CD86; and signal 3 is delivered by release of IL-12. Immature DCs receive a combination of maturation stimuli from different sources, which they integrate and translate into different qualities of adaptive T cell responses. Innate lymphocytes, which are already active before the onset of adaptive T cell responses, are crucial for DC maturation. For example, CD1c-restricted γδ T cells can drive DC differentiation toward a Th1-polarizing program (13). This bidirectional interaction between innate lymphocytes and DCs, called DC instruction, is mediated by cytokines, such as IFN-γ, and by direct cell-to-cell interaction through ligands of the TNF family, most notably CD40L (14, 15). Importantly, optimal IL-12 production, the signature cytokine of Th1 responses, requires conditioning of DCs by pathogen-associated danger signals that synergistically act through multiple pattern recognition receptors (PRRs) (16–19). To understand viral pathogenesis and develop effective viral vaccines, it is of the utmost importance to unravel the intricate interactions among DCs, innate lymphocytes, and pathogen-associated danger signals.

In this study, we analyzed the modulation of DC instruction by the VZV vaccine compared with virulent VZV clinical isolates. We show that the VZV vaccine primes human MDDCs for IL-12 production. In contrast, virulent VZV disrupts the programming of MDDCs toward a Th1-polarizing function. As an underlying mechanism, we identified a block in PRR signaling postinfection of MDDCs with virulent VZV, a fundamental immunological difference between the vaccine and clinical isolates. In conclusion, we define a novel virulence-associated immune-evasion strategy that is important for understanding VZV-associated pathogenesis and has to be considered in future attempts to develop novel vaccines.

Materials and Methods

Human samples

Skin punch biopsies (4 mm) were taken from control subjects undergoing breast reduction and from herpes zoster patients (vesicular stage, n = 2). Patients with herpes zoster were immunocompetent (mean age, 62 ± 10.5 y). Skin biopsies were embedded in Jung tissue freezing medium (Leica Instruments, Göttingen, Germany) and cryo-frozen. Five-micrometer sections were fixed for 10 min in acetone (−20°C), air-dried overnight, and stored at −80°C. Written informed consent for skin punch biopsies was obtained in accordance with ethical approval EA1/160/06 of the Charité—Universitätsmedizin Berlin.

Isolation and VZV infection of epidermal LCs and dermal DCs

Human skin was obtained from healthy volunteers undergoing plastic surgery and prepared as recently described (20). Epidermal sheets were detached from the dermis after overnight incubation with dispase I (2 U/ml; Roche Applied Science, Mannheim, Germany) at 4°C. After incubation with trypsin solution containing 0.25% trypsin in PBS with 5 mM MgCl2 and 10 μg/ml DNase (Roche Applied Science) for 15 min at 37°C, LCs were positively selected from single-cell suspensions using MACS and human CD1c (BDCA-1)† Dendritic Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The dermal pulp was incubated for 1.5 h at 37°C in PBS solution containing 10% FCS, penicillin/streptomycin (10,000 U/ml), amphotericin B (2.5 μg/ml), 5 mM MgSO4, 12 mg collagenase (Cell-Systems Biotechnologie Vertrieb, St. Katharinien, Germany), and 6.5 g hyaluronidase type I S (Sigma-Aldrich, Deisendorf, Germany). The filtered suspension was used to isolate dermal DCs with the human CD1c (BDCA-1)† Dendritic Cell Isolation Kit. LCs and dermal DCs were centrifuged at 160 × g for 45 min at room temperature on mock- or VZV-infected fibroblast monolayers with a density of 1 × 106 cells/ml in media containing recombinant human (rh)TGF-β (10 ng/ml), rhGM-CSF (500 IU/ml), and rIL-4 (200 IU/ml).

Viruses

VZV was propagated on human embryonic lung fibroblasts. VZV clinical isolates designated JoSt (genotype E1), M1_935/05 (genotype M1), E2_769/05 (genotype E2), and E1_667/05 (genotype E1) were isolated from varicella patients. All VZV strains used in this report were analyzed and genotyped by A. Sauerbrei, as previously reported (21). The vaccine strain V-Oka was grown from the live attenuated vaccine Varilrix (GlaxoSmithKline, Research Triangle Park, NC). The titers of VZV stocks (VZV-infected cells) were determined by calculation of the tissue culture infectious dose 50% using the Reed–Muench formula. CD40L-expressing fibroblasts were kindly provided by R.A. Kroczek (Robert Koch Institute, Berlin, Germany).

Generation and VZV infection of MDDCs

Buffy coats were obtained from the Deutsche Rote Kreuz (Dresden, Germany). Monocytes were isolated through negative selection by the MACS technique (Miltenyi Biotec) and differentiated within 3 d into immature MDDCs by culture with rhGM-CSF (500 IU/ml) and rIL-4 (200 IU/ml) (Immunotools, Friesoythe, Germany), as described previously (22). For VZV infection, immature DCs were centrifuged at 150 × g for 45 min at room temperature on a VZV-infected fibroblast monolayer. After 24 h, DCs were removed, and assays were performed 2 d after VZV infection. Mock DCs were set up as described above using uninfected fibroblasts.

Abs and reagents

Anti-CD1a (HI-49) was purchased from Immunotools; anti-CD1b (4.A7.6) and anti-CD1c (L161) were obtained from Immunotech (Hamburg, Germany); anti-CD40 (5C3), anti-CD83 (HB15e), anti-CD86 (IT2.2), anti-TCR-γδ1 (11F2), anti-IgG1 (MOPC-21), and anti-IgG2b (MPC-11) were purchased from BD Pharmingen (Heidelberg, Germany); and anti-glycoprotein E (gE) (MAB8661) was obtained from Chemicon International (Hoffheim, Germany). As secondary Abs, Alexa Fluor 488-labeled anti-IgG2a, and Alexa Fluor 568-labeled anti-IgG1 from Invitrogen (Karlsruhe, Germany) were used; FITC-, R-PE–, allophycocyanin-, and Cy5-labeled isotype-specific Abs from Jackson ImmunoResearch Laboratories (West Grove, PA) were used. For neutralization assays, mouse IgG1 (MOPC-21) and mouse anti-CD1c (L161) were added at 20 μg/ml, and polyclonal anti-IL-12 (R&D Systems, Wiesbaden, Germany) was added at 5 μg/ml. Stimulation with LPS was performed at 1 μg/ml and with PHA at 100 ng/ml. Apoptotic death was visualized by using FITC-labeled Annexin V and propidium iodide (PI; Santa Cruz Biotechnology, Santa Cruz, CA).

T cell assays

The well-characterized CD1c-restricted γδ T cell clone (JR.2.28) was cultured by periodic stimulation with PHA-P in the presence of irradiated EBV-transformed B cells and PBMCs, as described (23). For cytokine secretion, mock- or VZV-infected immature MDDCs were cocultured for 48 h with γδ T cells at an E/T ratio of 1:1 (1 × 103 cells/well). Supernatants were collected and stored at −20°C.

Measurement of cytokine production

Cytokine concentration of supernatants was measured by ELISA. ELISA kits for human IL-12p70 (eBioscience, Vienna, Austria) and IFN-γ (Immunotools) were used.

Immunohistochemistry

Skin sections (5 μm) were blocked with 5% goat serum (host species of the secondary Ab) for 1 h at room temperature in a humidified chamber and then were incubated with the specific primary Abs for 1 h at 37°C. Specific Ag was detected with Alexa Fluor 488-labeled anti-IgG3, and Alexa Fluor 568-labeled anti-IgG, as secondary Abs (1:400) by incubating for 45 min at room temperature. Nuclei were visualized by using the DNA-intercalating dye DAPI (1:20,000). Stained slides were analyzed using a BX60F-3 microscope with Cell D software (Olympus, Hamburg, Germany).

Flow cytometry

Cells were washed once in ice-cold FACS-wash solution (PBS with 1% FCS and 0.02% sodium azide) and incubated in ice-cold FACS-block solution (PBS with 10% FCS and 0.2% sodium azide) with primary Abs for 1 h. Cells were washed in ice-cold FACS-wash solution and stained
were found on a minor population (18% and 21%, respectively).

Viral gE at low density, whereas high amounts of this molecule same efficiency (Fig. 1F). The majority of cells (73%) expressed viral gE at low density, whereas high amounts of this molecule were found on a minor population (18% and 21%, respectively).

Statistical analysis

SPSS (Chicago, IL) and GraphPad Prism (GraphPad, San Diego, CA) software programs were used to perform statistics. The Wilcoxon test was used to determine the significance of differences between groups; p values <0.05 were considered significant (two-tailed). The actual p values are indicated in the box-and-whisker plots.

Results

Virulent VZV and avirulent V-Oka infect skin DC subsets with similar efficiency

First we assessed whether DC subsets found in human skin, the major replication site for VZV, show differential susceptibility to infection with virulent VZV and the avirulent VZV vaccine strain. For this purpose, LCs and dermal DCs were isolated from normal human skin. Surface staining for myeloid marker CD11c and langerin (LCs, Fig. 1A) or CD11c and CD1c (dermal DCs, Fig. 1C) confirmed the purity of the preparations. Freshly isolated LCs and dermal DCs were infected with VZV, and the surface expression of viral gE was determined. After mock infection, LCs and dermal DCs did not express gE. In contrast, 14% and 20%, respectively, of VZV-infected LCs (Fig. 1B) and 12% and 17%, respectively, of dermal DCs (Fig. 1D) strongly expressed gE after VZV infection. No differences in the efficiency of infection could be observed between the avirulent vaccine strain V-Oka and JoSt, a virulent VZV strain. Neither LCs nor dermal DCs upregulated costimulatory molecules postinfection (data not shown). Moreover, in vitro-generated MDDCs, which model DCs accumulating under inflammatory conditions, were susceptible to VZV infection, as previously described (10). V-Oka and JoSt induced similar DC clustering (Fig. 1E) and infected immature MDDCs with the same efficiency (Fig. 1F). The majority of cells (73%) expressed viral gE at low density, whereas high amounts of this molecule were found on a minor population (18% and 21%, respectively).

Taken together, these findings demonstrate that virulent VZV and V-Oka do not differ with regard to their capacity to infect skin DC subsets that are found during steady state or under inflammatory conditions.

Inflammatory DCs accumulate in herpes zoster lesions

Inflammatory DCs play an important role in the development of Th1-polarized T cells that produce IFN-γ and protect against viruses (24). Therefore, we determined whether inflammatory DCs occur during VZV-induced inflammation. For this purpose, we studied skin sections from papulovesicular lesions of herpes zoster patients. Staining with DAPI (Fig. 2A) or with Papanicolaou’s solution and eosin (data not shown) revealed a strong cellular infiltration. Using immunofluorescence microscopy, we localized myeloid CD11c-expressing DCs and assessed their CD1 expression while visualizing VZV infection by staining for viral gE. CD11c+ DCs strongly infiltrated VZV-infected skin lesions (Fig. 2B). In contrast, CD1a+ LCs disappeared from herpes zoster lesions and were found only in the epidermis of the control skin (Fig. 2C). The infiltrating DCs also expressed CD1b (Fig. 2D). DCs with detectable CD1c expression were mainly detected in close proximity to VZV-containing vesicles in inflamed skin tissue, but they were evenly distributed in the upper dermis of control skin sections (Fig. 2E). Probably because papulovesicular lesions represent an advanced stage of VZV infection in the dermis, viral gE was detectable only in a few CD1c+ cells (Fig. 2E). Altogether, these findings suggest that steady-state DCs disappear during viral spread, whereas inflammatory DCs strongly accumulate in herpes zoster lesions.

MDDCs infected with virulent VZV fail to stimulate IFN-γ secretion by innate cells

Next, we comparatively studied the impact of virulent VZV and V-Oka on phenotype and function of MDDCs in vitro. Because herpesviruses, including HSV (25, 26) and human CMV (22), can block CD1 expression on MDDCs, we investigated the effect of VZV on group 1 CD1 molecules. Although the expression of CD1a (Fig. 3A, 3B) and CD1b (Fig. 3A) remained unaltered, VZV infection with either strain significantly increased CD1c expression on immature MDDCs (Fig. 3A, 3B). This was not due to
an increased abundance of CD1c-encoding transcripts, suggesting that the underlying mechanism was operating on the protein level (data not shown). We used the CD1c-restricted V\(\delta\)1+ T cell clone JR.2.28 (23) to study the functional impact of VZV-induced CD1c upregulation. V\(\delta\) T cells expressing variable \(\delta\)1 chain (V\(\delta\)1+ T cells) represent the major V\(\delta\) lymphocyte population in human epithelial tissue. Strikingly, V-Oka–infected immature MDDCs stimulated CD1c-restricted V\(\delta\)1+ T cells to secrete large amounts of IFN-\(\gamma\) (Fig. 3C,3D). In contrast, JoSt-infected immature MDDCs failed to stimulate IFN-\(\gamma\) secretion by CD1c-restricted V\(\delta\)1+ T cells. Abs to CD1c, but not a respective isotype control, blocked IFN-\(\gamma\) secretion, showing that stimulation of V\(\delta\) T cells required CD1c (Fig. 3E). Altogether, these observations demonstrated a striking functional difference between immature MDDCs infected with the vaccine strain V-Oka and those infected with the VZV clinical isolate JoSt.

**Virulent VZV and the avirulent VZV vaccine strain infect \(\gamma\)\(\delta\) T cells**

In the previous experiments, we could not exclude that virulent VZV directly targets the function of innate lymphocytes. Therefore, we investigated whether VZV is transmitted to CD1c-restricted \(\gamma\)\(\delta\) T cells. For this purpose, the expression of viral gE on \(\gamma\)\(\delta\) T cells after coculture with VZV-infected MDDCs was quantified (Fig. 4A). Avirulent V-Oka and clinical isolate JoSt were transmitted from infected immature MDDCs to \(\gamma\)\(\delta\) T cells with the same low efficiency (6–7\%). However, IFN-\(\gamma\) secretion by \(\gamma\)\(\delta\) T cells in the presence of JoSt-infected immature MDDCs could be restored by stimulation with PHA, demonstrating that virulent VZV does not destroy the functional integrity of \(\gamma\)\(\delta\) T cells (Fig. 4B). In conclusion, the block of IFN-\(\gamma\) release by innate lymphocytes after stimulation with JoSt-infected immature MDDCs was due to a virus-induced defect in MDDCs.

**Interference of virulent VZV with DC function is independent of its genotype**

We next tested whether the failure of JoSt-infected immature DCs to activate CD1c-restricted innate cells is a general characteristic of virulent VZV strains. To this end, we studied the currently circulating European VZV strains that are grouped into the main genotypes M1, E1, and E2 (21). According to phylogenetic analysis, the isolate M1_935/05 belonged to genotype M1, the isolate E2_769/05 belonged to genotype E1, and the isolates E2_769/05 and JoSt clustered within genotype E1 (Fig. 5A). These VZV strains grew equally well in human embryonic lung fibroblasts (data not shown). Intriguingly, all VZV clinical isolates, regardless of their genotype, strongly blocked the capacity of immature MDDCs to activate CD1c-restricted \(\gamma\)\(\delta\) T cells (Fig. 5B). These functional differences could have been due to differential VZV-induced apoptosis of immature MDDCs. To exclude this...
VZV-induced apoptosis in 18–26% of immature MDDCs, regardless of infected immature MDDCs contained cocultured with immature MDDCs 2 d after mock, V-Oka, or JoSt infection. One of six independent experiments done in triplicate is shown (error bars are ± 1 SD). D, Box-and whisker plot of MFI of CD1a-restricted γδ T cells cocultured with immature MDDCs 2 d after mock, V-Oka, or JoSt infection (n = 6). E, Anti-CD1c Abs block IFN-γ secretion by CD1c-restricted γδ T cells cocultured with immature MDDCs 2 d after mock, V-Oka, or JoSt infection. Anti-CD1c Abs or the respective isotype control were used at 20 μg/ml. Data are representative of two independent experiments done in triplicate.

possibility, VZV-infected immature MDDCs were stained with FITC-Annexin V and PI and analyzed by flow cytometry (Fig. 5C). Mock-infected immature MDDCs contained <3% Annexin V single-positive (apoptotic) cells and <1% double-positive (necrotic) cells. By contrast, VZV induced apoptosis in 18–26% of immature MDDCs, regardless of whether they were infected with the vaccine or VZV clinical isolates. In all cases, the percentage of necrotic cells was <5%. Moreover, there was no difference between V-Oka and virulent VZV with regard to the infection rate, as determined by viral gE expression on MDDCs (Fig. 5D). Thus, the functional difference between MDDCs infected with V-Oka and MDDCs infected with clinical isolates was not due to different survival rates of infected DCs or different infection rates. In conclusion, virulent VZV, irrespective of its genotype and geographical origin, interferes with the capacity of immature DCs to stimulate IFN-γ secretion by CD1c-restricted γδ T cells.

DCs infected with virulent VZV are resistant to Th1-promoting instruction

Innate lymphocytes, such as CD1c-restricted γδ T cells, confer on immature MDDCs the capacity to efficiently prime Th1 cells, a process called DC instruction (13, 15, 27). We investigated whether VZV-infected immature MDDCs were still susceptible to instruction by CD1c-restricted γδ T cells. For this purpose, the expression of CD83 (Fig. 6A) and CD86 (Fig. 6B) on VZV-infected immature MDDCs was determined in the absence or presence of γδ T cells. As a control, immature MDDCs were treated with LPS. In the absence of γδ T cells, VZV-infected immature MDDCs did not show a mature phenotype. Surprisingly, immature MDDCs, whether infected with the vaccine V-Oka or the clinical isolate JoSt, phenotypically matured after adding γδ T cells or LPS. We next studied the ability of CD1c-restricted γδ T cells to induce VZV-infected immature MDDCs to secrete IL-12, a key cytokine for the stimulation of Th1 cell responses (Fig. 6C). In the absence of γδ T cells, neither mock-infected nor VZV-infected immature MDDCs secreted bioactive IL-12p70. Strikingly, in the presence of γδ T cells, immature MDDCs infected with vaccine strain V-Oka secreted increased amounts of IL-12p70 into the supernatant compared with mock-infected MDDCs. By contrast, immature MDDCs infected with VZV clinical isolates and cocultured with γδ T cells failed to produce significant amounts of IL-12p70. Similar to the European VZV strains, a Japanese VZV clinical isolate belonging to the genotype J, like V-Oka, blocked the induction of IL-12 release in cocultures of MDDCs with γδ T cells (data not shown). To assess the relevance of IL-12 by MDDCs on IFN-γ secretion by γδ T cells, we added neutralizing IL-12 Abs to the cultures. The addition of neutralizing IL-12 Abs blocked >50% of IFN-γ secretion by γδ T cells when cultured with V-Oka–infected immature MDDCs (Fig. 6D). These results indicated that the VZV vaccine and virulent VZV allow phenotypic maturation of immature MDDCs upon cross-talk with innate lymphocytes. Strikingly, however, only the VZV vaccine promotes functional instruction of MDDCs, leading to the release of Th1-polarizing IL-12.

Viral transmission from VZV-infected DCs to γδ T cells. CD1c-restricted γδ T cells were cocultured with immature MDDCs 2 d after mock, V-Oka, or JoSt infection. A, The T cell population was gated according to the FSC/SSC profile and analyzed for the coexpression of γδ TCR and the viral gE (percentage of cells is indicated in each quadrant). Data are representative of three independent experiments. B, IFN-γ secretion by CD1c-restricted γδ T cells cocultured with VZV-infected immature MDDCs after stimulation with PHA (100 ng/ml). One of three independent experiments done in triplicate is shown (error bars are ± 1 SD).

Next, we analyzed whether disruption of DC instruction by virulent VZV was due to a viral block in CD1c-restricted γδ T cells. As a control, immature MDDCs were treated with LPS. In the absence of γδ T cells, VZV-infected immature MDDCs did not show a mature phenotype. Surprisingly, immature MDDCs, whether infected with the vaccine V-Oka or the clinical isolate JoSt, phenotypically matured after adding γδ T cells or LPS. We next studied the ability of CD1c-restricted γδ T cells to induce VZV-infected immature MDDCs to secrete IL-12, a key cytokine for the stimulation of Th1 cell responses (Fig. 6C). In the absence of γδ T cells, neither mock-infected nor VZV-infected immature MDDCs secreted bioactive IL-12p70. Strikingly, in the presence of γδ T cells, immature MDDCs infected with vaccine strain V-Oka secreted increased amounts of IL-12p70 into the supernatant compared with mock-infected MDDCs. By contrast, immature MDDCs infected with VZV clinical isolates and cocultured with γδ T cells failed to produce significant amounts of IL-12p70. Similar to the European VZV strains, a Japanese VZV clinical isolate belonging to the genotype J, like V-Oka, blocked the induction of IL-12 release in cocultures of MDDCs with γδ T cells (data not shown). To assess the relevance of IL-12 by MDDCs on IFN-γ secretion by γδ T cells, we added neutralizing IL-12 Abs to the cultures. The addition of neutralizing IL-12 Abs blocked >50% of IFN-γ secretion by γδ T cells when cultured with V-Oka–infected immature MDDCs (Fig. 6D). These results indicated that the VZV vaccine and virulent VZV allow phenotypic maturation of immature MDDCs upon cross-talk with innate lymphocytes. Strikingly, however, only the VZV vaccine promotes functional instruction of MDDCs, leading to the release of Th1-polarizing IL-12.
compared with V-Oka–infected immature MDDCs (data not shown). However, after stimulation with CD40L-expressing fibroblasts, JoSt-infected and V-Oka–infected immature MDDCs secreted more IL-12p70 than mock-infected immature MDDCs (Fig. 7C). This increased IL-12 release was mirrored by enhanced CD40L-mediated activation of the MAPK pathway in VZV-infected DCs compared with mock-infected DCs (data not shown). Next, γδ T cells were added to the cultures, and IFN-γ secretion was measured (Fig. 7D). Intriguingly, the ability of JoSt-infected immature DCs to stimulate IFN-γ secretion by γδ T cells was fully rescued in the presence of CD40L-expressing fibroblasts. Collectively, these data demonstrate that despite blocking CD40 upregulation on
infected DCs, virulent VZV amplifies CD40 signaling, similar to the VZV vaccine.

**Virulent VZV selectively inhibits TLR2 signaling**

Next, we investigated DC priming by signaling through TLRs, a prerequisite of DC instruction (13, 29). In the absence of innate cells, signaling through multiple PRRs can synergize to efficiently prime IL-12 secretion by DCs (17–19). Therefore, we tested whether VZV can cooperate with signaling through TLR2 and TLR3, which are involved in the detection of HSV and VZV, the human alphaherpesviruses (30–33). They are located on the cell surface (TLR2) and endosomes (TLR3). TLR2 recognizes viral glycoproteins in the viral envelope, whereas TLR3 detects dsRNA. VZV-infected immature DCs were challenged with lipoteichoic acid (LTA), a TLR2 agonist, or polyinosinic:polycytidylic acid [poly(I:C)], a TLR3 agonist. As shown in Fig. 8A, uninfected or mock-infected DCs treated with LTA did not secrete IL-12p70. Intriguingly, DCs infected with V-Oka or HSV released large amounts of IL-12p70 after challenge with LTA. In sharp contrast, DCs infected with JoSt, the virulent VZV strain, secreted minimal amounts of IL-12 in response to LTA. Strikingly, efficient priming of DCs required viral replication, because LTA did not synergize with ultraviolet-inactivated virus to induce the release of IL-12. Similar to LTA, the TLR3 agonist poly(I:C) could not induce uninfected or mock-infected DCs to secrete substantial amounts of IL-12 (Fig. 8B). However, V-Oka and, to a lesser extent, virulent VZV, but not HSV, cooperated with poly(I:C) to trigger IL-12 secretion. DCs infected with ultraviolet-inactivated virus did not respond to poly(I:C), as observed for LTA. Taken together, these data demonstrated that replicating VZV can prime DCs for IL-12 release. However, there was a fundamental difference between V-Oka and virulent VZV. The former synergized with TLR2 and TLR3, whereas the latter selectively blocked signaling through TLR2, which is known to detect virulent VZV.

**Discussion**

In this study, we demonstrated the immunological difference between V-Oka, the vaccine strain, and VZV clinical isolates: induction of inflammatory DCs toward a Th1-polarizing program is promoted by V-Oka but disrupted by virulent VZV.

We showed that different DC subsets are susceptible to VZV infection. LCs and dermal DCs isolated from normal human skin could be infected by VZV. Other investigators demonstrated recently that plasmacytoid DCs isolated from human blood and LCs generated from MUTZ-3 cells are permissive to VZV infection (34). Additionally, immature DCs generated in vitro from monocytes were susceptible to VZV infection, as previously reported (10, 11). The majority of these MDDCs expressed viral gE at low levels; only 10–20% showed strong surface expression of this molecule. The distinct peaks can be explained by the highly cell-associated nature of VZV. For VZV infection, MDDCs have to be centrifuged on a VZV-infected fibroblast monolayer. During the first round of virus transmission, the virus infects only a few MDDCs because of the close cell-to-cell contact required. In this induction phase, innate responses are weak, and the virus multiplies efficiently, resulting in strong viral gE surface expression. In contrast, most MDDCs are probably infected later when the innate defense is already fully established in the cell culture, resulting in less efficient viral replication and low viral gE surface expression. Importantly, however, there was no difference with regard to the infection efficiency between the VZV vaccine and virulent VZV.

Skin biopsies from zoster lesions revealed that LCs disappear in the vicinity of virus-containing vesicles, confirming other reports (34, 35). This could have been due to virus-induced apoptosis. Alternatively, VZV-infected LCs could have emigrated to the regional lymph nodes to transfer VZV Ag to resident DCs. In contrast, inflammatory DCs strongly infiltrated VZV-infected skin. Similar observations were made for lepromatous skin lesions in humans (8) and in an experimental mouse model of leishmanial infection in the dermis (9). It is likely that the majority of VZV-infected inflammatory DCs had already undergone apoptosis or migrated to the lymph nodes at the time when the skin punch biopsies were taken. This could explain the scarcity of CD1c+ cells expressing viral gE in papulovesicular lesions, a relatively late stage of VZV infection in the dermis. Alternatively, VZV-infected inflammatory DCs might be difficult to detect in skin sections. This assumption is supported by the fact that most
VIRAL DISRUPTION OF DENDRITIC CELL INSTRUCTION

MDDCs infected in vitro with VZV show only low gE expression in FACS analysis.

The infiltrating DCs are derived from monocytes that are recruited to the site of inflammation and resemble dermal DCs found in normal skin. After maturation, they migrate to the regional lymph nodes to induce a protective Th1 cell response (36). Recently, it was demonstrated in the mouse model that inflammatory DCs are required to induce a Th1-polarized response that protects against viruses (24). For this reason, we modeled inflammatory DCs in vitro and analyzed in detail whether VZV can interfere with their ability to couple innate to adaptive immune responses.

In accordance with a previous report, no changes in CD1a and CD86 expression were detected postinfection of immature DCs with JoSt, a VZV clinical isolate (10). Surprisingly, vaccine and VZV clinical isolates significantly enhanced CD1c expression on immature DCs. Although CD1c can present synthetic lipopeptides that mimic N-terminally acylated peptides found in cellular and viral proteins (37), the self ligand and possible viral ligands recognized by CD1c-restricted γδ T cells are still unknown (23). Strikingly, V-Oka–infected DCs, but not immature DCs infected with VZV clinical isolates, could efficiently stimulate IFN-γ release by CD1c-restricted γδ T cells. In addition, VZV was demonstrated to interfere with IFN-γ signal transduction via the Jak/Stat pathway (38). Such mechanisms aiming at IFN-γ release or IFN-γ signal transduction represent an important viral defense strategy. First, IFN-γ is a key cytokine polarizing the adaptive immune response toward Th1 lymphocytes, which provide help for cytotoxic T cells and can themselves lyse virus-infected cells. Second, a rapid supply of IFN-γ in an early phase of VZV infection could prevent viral spread and skin lesions, through noncytolytic virus clearance (39).

Viral pathogens have developed multiple mechanisms that eliminate DCs (40). In our study, VZV clinical isolates and the VZV vaccine induced apoptosis to a similar extent: 18–26% of DCs underwent programmed cell death postinfection. Thus, the lack of IFN-γ release during cross-talk of DCs infected with virulent VZV and immune cells was not due to enhanced apoptosis induction. Apoptotic debris from VZV-infected immature DCs could be phagocytosed by uninfected bystander DCs that subsequently mature, migrate to the regional lymph nodes, and cross-present VZV-derived peptides to conventional αβ T lymphocytes. Such a mechanism could explain why high frequencies of VZV-specific αβ T cells are observed in immunocompetent individuals after natural VZV infection (41), despite multiple and highly efficient viral immune-evasion mechanisms. In contrast, it is also possible that viruses, such as VZV, trigger DC apoptosis to blunt the antiviral immune response because apoptotic blebs may constitute a tolerogenic signal (42).

VZV is known to possess T cell tropism and can be transferred from infected DCs to αβ T cells (38). Infected αβ T lymphocytes in peripheral blood transport VZV to the skin during the cell-associated viremia of primary infection (2). We found that infected DCs transmit VZV to CD1c-restricted γδ T cells, suggesting that these intraepithelial lymphocytes contribute to viral dissemination in the skin during reactivation of VZV from sensory ganglia. However, infection with virulent VZV did not silence CD1c-restricted γδ T cells, suggesting that the pathogen disrupts DC instruction at the site of DCs.

Upon interaction with innate lymphocytes, DCs upregulate the surface expression of CD83 and CD86, secrete IL-12, and efficiently induce Th1-like adaptive immune responses (13, 15, 28, 29, 43–46). We observed that in the presence of γδ T cells, DCs acquired a fully mature phenotype, whether infected with the vaccine V-Oka or the clinical isolate JoSt. Consequently, the inability of DCs infected with virulent VZV strains to stimulate IFN-γ secretion by CD1c-restricted γδ T cells was not due to a viral block in upregulation of costimulatory molecules (signal 2). However, only the VZV vaccine licensed release of IL-12 (signal 3), a potent inducer of IFN-γ secretion from T cells and of Th1 cell differentiation. By contrast, all DCs infected with VZV clinical isolates, regardless of their genotype, were unable to secrete significant amounts of IL-12 upon interaction with CD1c-restricted γδ T cells. Neutralization of IL-12 by Abs during coculture of V-Oka–infected DCs with CD1c-restricted γδ T cells decreased IFN-γ secretion 60–70%. These results emphasize that DC instruction is a bidirectional process, whereby DCs and innate lymphocytes stimulate each other (43, 47).

In addition to IFN-γ, a second signal provided by CD40 engagement is required for triggering IL-12 secretion by DCs (48, 49). We observed a moderate, although significant, increase in CD40 on vaccine strain-infected DCs, whereas DCs infected with virulent VZV did not show this effect. However, this difference in CD40 surface expression on infected DCs was functionally irrelevant. In comparison with unstimulated control cells, JoSt-infected and V-Oka–infected DCs released large amounts of IL-12 after stimulation with CD40L-expressing cells. Remarkably, the CD40L-induced IL-12 secretion by VZV-infected DCs was even greater than the amount of IL-12 measured after stimulation of mock-infected DCs. This result supports the concept that IL-12 release triggered by CD40–CD40L interaction is amplified by pathogen-derived danger signals (19). Additionally, analysis of the CD40 signaling pathway in VZV-infected DCs cocultured with CD40L-expressing cells revealed enhanced phosphorylation of JNK, ERK, and p38 kinases compared with mock-infected DCs (data not shown). In this regard, DCs infected with virulent VZV differ from HSV-infected DCs, which do not produce IL-12 in response to CD40L-expressing cells (50). Finally, IFN-γ secretion by the cocultured γδ T cells could be fully rescued after stimulating virulent VZV-infected DCs with CD40L-expressing cells. These data clearly showed that virulent VZV synergizes with CD40 signaling as efficiently as the VZV vaccine and does not target CD40 signaling to interfere with DC instruction.

There was the possibility that virulent VZV subverts priming of DCs for optimal IL-12 production, which requires synergetic signaling through multiple PRRs (17, 18). Multiple PPRs at different cellular locations are involved in the detection of alphaherpesviruses, such as VZV and HSV. Viral glycoproteins within the viral envelope are recognized by TLR2 at the cell surface (30–32). In contrast, HSV-infected DCs, which do not produce IL-12 in response to CD40L-expressing cells (50). Finally, IFN-γ secretion by the cocultured γδ T cells could be fully rescued after stimulating virulent VZV-infected DCs with CD40L-expressing cells. These data clearly showed that virulent VZV synergizes with CD40 signaling as efficiently as the VZV vaccine and does not target CD40 signaling to interfere with DC instruction.

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dissemination of HSV (33). In conclusion, our results suggest that virulent VZV actively interferes with signaling downstream of surface TLR2. It was shown that vaccinia virus and hepatitis C virus encode proteins that block intracellular TLR signaling components (53, 54). Similarly, virulent VZV might encode a TLR antagonist that disrupts intracellular TLR2 signaling.

The molecular mechanisms underlying attenuation of the VZV vaccine are not understood. Our results showed that virulent VZV disrupts instruction of DCs toward a Th1-polarizing signal. During DC instruction by innate cells, virulent VZV strongly blocked the release of IL-12 (signal 3). As an underlying mechanism, we identified a viral block of synergistic signaling through TLR2. This block is essential for the induction of an efficient antiviral immune response that prevents disease.

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Disclosures
The authors have no financial conflicts of interest.

References


