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Varicella-Zoster Virus Activates Inflammatory Cytokines in Human Monocytes and Macrophages via Toll-Like Receptor 2

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The pattern recognition receptor Toll-like receptor 2 (TLR2) has been implicated in the response to several human viruses, including herpes simplex viruses (types 1 and 2) and cytomegalovirus. We demonstrated that varicella-zoster virus (VZV) activates inflammatory cytokines in cell lines expressing TLR2. VZV specifically induced interleukin-6 (IL-6) in human monocytes via TLR2-dependent activation of NF-κB, and small interfering RNA designed to suppress TLR2 mRNA reduced the IL-6 response to VZV in human monocyte-derived macrophages. Unlike other herpesviruses, the cytokine response to VZV was species specific. VZV did not induce cytokines in murine embryonic fibroblasts or in a mouse cell line, although VZV did activate NF-κB in a human cell line expressing a murine TLR2 construct. Together, these results suggest that TLR2 may play a role in the inflammatory response to VZV infection.

Varicella-zoster virus (VZV) is a pathogen that causes two distinct clinical syndromes: varicella (chicken pox) as the primary infection and herpes zoster (shingles) upon reactivation of VZV in sensory neurons. VZV infects human mucosal surfaces, followed by systemic viremia with spread to skin and subsequent establishment of latency in neurons of cranial and spinal dorsal root ganglia. Induction of the innate immune response occurs during acute infection, which is characterized by the release of alpha interferon (IFN-α) and IFN-γ (1). Within a few days, cell-mediated and humoral immune responses develop.

Toll-like receptors (TLRs) are a key component of the host innate recognition system. At least 11 TLRs are expressed in humans (25). Specifically, TLR2 interacts with viruses or their components, including measles virus, hepatitis C virus, and several herpesviruses, including cytomegalovirus and herpes simplex virus (HSV) (2, 3, 4, 15). Activation of TLR2 by these viruses, including some herpesviruses, is followed by the production of inflammatory cytokines, including interleukin-1 (IL-1), IL-8, and tumor necrosis factor alpha (TNF-α). In addition, the induction of IFN-α by HSV involves TLR9 (11, 17).

VZV DNA can be detected in peripheral blood mononuclear cells (PBMC) during viremia (18), and monocytes have been shown by reverse transcription-PCR to support productive infection of VZV (10). VZV infection is associated with induction of inflammatory cytokines, including IL-1α production by VZV-infected epithelial cells (12). Human mononuclear cells have been shown to produce TNF-α in vitro in response to VZV-infected fibroblasts (21). Evidence that VZV infection activates monocytes to produce inflammatory cytokines is the hallmark of a TLR-dependent innate immune response.

Human monocytes and monocyte-derived macrophages normally express TLR2 and produce cytokines such as IL-1, TNF-α, and IL-6 in response to stimulation with TLR2 ligands, including viruses. The present work demonstrates that TLR2 contributes to human inflammatory cytokine production during VZV infection.

MATERIALS AND METHODS

Virus preparation. A clinical isolate of VZV was cultured in human embryonic lung fibroblasts (HEL). HELF were grown in Dulbecco’s modified Eagle’s medium (Gibco, Gaithersburg, MD) supplemented with 2% heat-inactivated fetal calf serum (HyClone, Logan, UT). PFU (1 × 10^6) of stock VZV was added to confluent HELF in a T-175 flask and removed after 2 h, and fresh culture medium was added. When cytopathic effect reached 60 to 70% (72 to 96 h postinfection), cells were harvested by trypsinization. VZV-infected HELF or uninfected control HELF were stored at 1.5 × 10^7 HELF/ml at −80°C in tissue culture medium with 10% dimethyl sulfoxide. Quantification was performed by plaque assay, VZV stocks contained 2 × 10^6 to 4 × 10^6 PFU/ml. UV irradiation of VZV-infected HELF for 60 min completely abrogated production of plaques. A clinical respiratory syncytial virus (RSV) isolate was also cultivated in HELF as a control for cellular proteins associated with a cytopathic virus. When cytopathic effect reached 60 to 70%, cells were harvested and stored at −80°C at a concentration of 1 × 10^7 PFU/ml.

Reagents and cells. Human embryonic kidney (HEK) 293 cells expressing human TLR2 and CD14 were cloned as described previously (14). HEK cells expressing human TLR9 and TLR4, murine RAW 264.7 cells expressing human TLR2, and plasmid constructs containing human or mouse TLR2 were gifts from E. Latz, E. Lien, and D. Golenbock (University of Massachusetts Medical School). Cells were transfected with an NF-κB firefly luciferase reporter plasmid and a control Renilla reniformis luciferase plasmid by using a GeneJuice (Novagen) transfection reagent. Human PBMC were isolated from haleinized blood by Percoll gradients (Matrix, Hudson, NH), and monocytes were purified by differential adherence to fibronectin. Monocytes were seeded into 96-well plates at a density of 5 × 10^5 cells/well or in 24-well plates at 5 × 10^5 cells/well in RPMI (Gibco) with 18% autologous serum. For some experiments, cells were allowed to differentiate into macrophages for 7 days. Mice deficient in TLR2 were generously provided by Shizuo Akira. Murine embryonic fibroblasts (MEF) were isolated as previously described (16). Acyclovir and polymyxin B were purchased from Sigma-Aldrich (St. Louis, MO).

TLR and non-TLR ligands. Lipopolysaccharide (LPS) from Escherichia coli serotype O11:B4 (Sigma, St. Louis, MO) was phenol extracted to remove

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12658
contaminating lipopeptides (8). Pam3CSK4 and Pam4CSK4 were purchased from EMC Microcollections (Tübingen, Germany). CpG 2006 DNA was purchased from Coley Pharmaceutical (Kanata, Ontario, Canada). IL-1β was purchased from R&D Systems (Minneapolis, MN) and TNF-α from Peprotech (Rocky Hill, NJ).

siRNA preparation. Twenty-one-nucleotide double-stranded RNAs were chemically synthesized as annealed oligonucleotides by Ambion (Austin, TX). Small interfering RNAs (siRNAs) were stored in water at −20°C. Sequences were predesigned by Ambion. The siRNA sequences used were as follows: for TLR2, 5′-GGC UUC UCU GUU UUG UGC CAA ddTdT-3′, and for TLR8, 5′-GGG AGU UAC UGC UUG AAG A ddTdT-3′.

Transfection of siRNA. Adherent human monoocyte-derived macrophages were transfected with 60 pmol siRNA per 5 × 106 cells and 3 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in serum-free Dulbecco’s modified Eagle’s medium for 4 h, rinsed, and then placed in fresh RPMI supplemented with 18% autologous serum.

ELISA. Human IL-6 and human IL-8 were measured by Quantikine enzyme-linked immunosorbent assay (ELISA) from R&D Systems (Minneapolis, MN). Murine IL-6 and murine TNF-α were assayed using OptEIA (BD Biosciences, Franklin Lakes, NJ).

Flow cytometry. TLR2 expression on the surface of human monoocyte-derived macrophages was determined by flow cytometry. Cells were detached by using 5 mM EDTA in phosphate-buffered saline. To block nonspecific antibody binding, cells were incubated with blocking buffer (phosphate-buffered saline with 20% serum from human whole blood, male type AB [Sigma]) for 60 min on ice (20). Cells were stained with anti-TLR2 monoclonal antibody (clone 2.1) labeled with allophycocyanin (APC) (eBioscience, San Diego, CA) or with isotype control antibody with APC (eBioscience, San Diego, CA) for 30 min on ice. Cells were analyzed using a FACSan analyzer. For each assay condition, at least 10,000 cells were analyzed.

RESULTS

VZV activation of NF-κB requires TLR2 and CD14. We examined the ability of human cells with or without TLRs on their surface to respond to cell-associated VZV. We used human HEK293 cells stably transfected with TLR2 and its coreceptor, the glycosylphosphatidylinositol (GPI)-anchored protein CD14 (HEK/TLR2/CD14). These cells were transiently transfected with an NF-κB luciferase reporter plasmid and a Renilla luciferase plasmid for internal control. A 6-h challenge revealed that cell-associated VZV activated NF-κB through TLR2 (Fig. 1A). UV irradiation of VZV-infected HELF did not significantly reduce its ability to activate NF-κB. Uninfected control HELF did not activate NF-κB in HEK/TLR2/CD14 cells. Positive controls included Pam3CSK4, a TLR2 ligand, and IL-1β, a TLR-independent activator of NF-κB. To ensure that the induction of NF-κB by VZV-infected HELF was not related to viral-induced cytopathic effects in HELF, RSV-infected HELF served as an additional control. VZV-infected HELF induced NF-κB to a 10-fold-greater degree than did cytopathic effect-matched RSV-infected HELF in HEK/TLR2/CD14 cells. Mycoplasma, a TLR2 ligand, was not detected in VZV-infected HELF by PCR studies (mycoplasma detection kit; American Type Culture Collection, Manassas, VA), which excluded this potential contaminant as a cause of the observed increase in NF-κB activation. In additional experiments, we used HEK expressing TLR2 without CD14 (HEK/TLR2). NF-κB activation by VZV-infected HELF was greatly enhanced after transfection and expression of the human CD14 plasmid but not with the control plasmid, suggesting that CD14 augments the recognition of VZV as a TLR2 ligand (Fig. 1B). As expected, TLR2-independent activation of NF-κB by IL-1β was not augmented by the presence of CD14.

HSV, another alpha-herpesvirus, interacts with TLR9 to induce IFN-α in dendritic cells (11, 17). To investigate a possible interaction between TLR9 and VZV, we tested the capacity of cell-associated VZV to induce NF-κB in HEK293 cells stably transfected with TLR9 (HEK/TLR9). Addition of VZV-infected HELF did not activate NF-κB in these cells (Fig. 1C). CpG DNA served as a positive control activator of TLR9, and TNF-α served as a TLR-independent positive control. Since TLR4 also interacts with viruses (13, 19), we tested the effects of VZV on HEK293 cells transfected with TLR4 and MD2, an endotoxin coreceptor. We found that NF-κB was not specifically induced by VZV in HEK/TLR4/MD2 cells (Fig. 1D). LPS and IL-1β were used for positive controls. Data from this assay also indicate that the VZV and HELF stocks do not contain LPS.

While UV irradiation of VZV did not appear to influence its ability to induce NF-κB in HEK/TLR2/CD14 cells, we wanted to confirm that TLR2-mediated induction of NF-κB by VZV-infected HELF does not require synthesis of new viral DNA. HEK/TLR2/CD14 cells were pretreated with acyclovir at 100 μg/ml for 30 min and then challenged with VZV-infected cells in the presence of acyclovir, which inhibits the viral DNA polymerase. The induction of NF-κB was identical to that by VZV-infected HELF without acyclovir in HEK/TLR2/CD14 cells (Fig. 1E). The activity of acyclovir was confirmed by plaque inhibition assay (data not shown). In addition, NF-κB was induced with VZV-infected HELF that had been frozen and thawed repeatedly. This diminishes the plaque titer of VZV-infected cells more than 1,000-fold (M. Levin, unpublished observation). Again, Pam3CSK4 and IL-1β served as positive controls.

VZV induces inflammatory cytokines in human PBMC and monocytes but not in murine cells. Acute VZV infection is associated with an inflammatory response characterized by fever and cytokine production (1, 21). Human monocytes, which are infected by VZV during acute infection, express TLR2 (3, 10). We challenged human monocytes with VZV-infected cells. Challenge with VZV-infected HELF was associated with the production of the cytokines IL-6 and IL-8 in a dose-dependent manner, while addition of control uninfected HELF was associated with minimal induction of IL-6 and IL-8 (Fig. 2A). Monocytes, which also express TLR4, secreted cytokines in response to LPS as expected. We could not attribute cytokine induction by VZV-infected HELF to LPS contamination, since the VZV-infected HELF did not induce NF-κB in HEK/TLR4/MD2 cells (Fig. 1D). Furthermore, we treated some monocytes with polymyxin B (10 μg/ml) for 30 min prior to the addition of and in the presence of stimuli, which resulted in a reduction of the cytokine response to LPS by greater than 90% but did not affect the response to VZV-infected HELF (Fig. 2A).

In contrast to HSV, which can infect and replicate in many types of cultured cells, VZV has a limited host range. To test whether VZV induces inflammatory cytokines in mouse cells, we stimulated MEF with VZV. MEF naturally express high levels of many TLRs, including TLR2, and produce cytokines such as IL-6 and MCP-1 in response to stimulation with TLR ligands (including the TLR2 ligand Pam3CSK4) (16). VZV-infected HELF did not induce cytokines in wild-type MEF, whereas Pam3CSK4 induced IL-6 in cells from wild-type but not TLR2 knockout mice in a dose-dependent manner, as
FIG. 1. Cell-associated VZV is a specific ligand for TLR2. (A) HEK293 cells expressing human TLR2 plus CD14 were transfected with an NF-κB-driven firefly luciferase reporter plasmid and a Renilla luciferase reporter plasmid and were stimulated for 6 h with VZV-infected HELF, UV-irradiated VZV-infected HELF, uninfected HELF, or RSV-infected HELF at 1:10 or 1:100 (ratio of HELF to HEK). Pam3CSK4 (100 ng/ml) and TNF-α (100 ng/ml) were used as positive controls. (B) HEK293 cells expressing human TLR2 were transfected with reporter plasmids and either a human CD14 plasmid or a pcDNA3.1 control plasmid. Cells were stimulated with VZV-infected HELF or uninfected HELF. Pam3CSK4 (100 ng/ml) was used as a CD14-enhanced, TLR2-dependent positive control. IL-1β (100 ng/ml) served as a CD14-independent, TLR2-independent activator of NF-κB. (C) HEK293 cells expressing human TLR9 were transfected with reporter plasmids and then challenged with VZV-infected or uninfected HELF. CpG DNA (3 μM) acted as a TLR9-dependent activator of NF-κB, and TNF-α (100 ng/ml) was a TLR-independent activator of NF-κB. Pam3CSK4 (100 ng/ml) or medium alone served as a negative control. (D) HEK293 cells expressing human TLR4 plus MD2 were transfected with reporter plasmids and subsequently challenged with VZV-infected or uninfected HELF, LPS (10 ng/ml), or IL-1β (100 ng/ml). Pam3CSK4 (100 ng/ml) or medium alone served as a negative control. (E) HEK293 cells expressing human TLR2 plus CD14 were transfected with reporter plasmids. Prior to stimulation, acyclovir was added at 100 μg/ml for 60 min. Cells were then stimulated with VZV-infected or uninfected HELF or controls for 6 h in the presence of acyclovir. Bars represent standard errors of the means for triplicate wells. RLU, relative light units.
expected (Fig. 2B). LPS was used as a TLR4-dependent, TLR2-independent positive control. Murine peritoneal exudate cells also failed to produce cytokines in response to VZV, even though these cells produce IL-6 in response to the TLR2 ligands zymosan and Pam3CSK4 (data not shown).

In a further attempt to characterize the murine response to VZV, we used RAW 264.7 cells, a mouse macrophage-like cell line that constitutively expresses murine TLR2 and CD14. To determine if the presence of human TLR2 could allow VZV to induce cytokines in murine cells, we used RAW 264.7 cells that were stably transfected with a human TLR2-yellow fluorescent protein (YFP) construct (RAW/hTLR2). Expression of this
FIG. 2—Continued.
construct was confirmed by examining YFP expression with confocal microscopy (data not shown). RAW/hTLR2 cells were not able to produce the NF-κB-mediated cytokine TNF-α in the presence of VZV-infected HELF after 24 h (Fig. 2C), although they were able to produce TNF-α in response to the positive controls Pam3CSK4 and LPS. We were thus unable to use TLR2 knockout mice for additional VZV studies.

**VZV is recognized by either human or murine TLR2.** Although human and mouse TLR2 share 70% amino acid identity by comparative amino acid sequence analysis, murine TLR2 recognizes specific peptides in a species-specific manner (5). To determine if VZV-infected HELF specifically activates human but not mouse cells due to differences in the TLR2 molecules themselves or due to the contribution of a human non-TLR cofactor, we transiently transfected HEK/CD14 cells with either murine or human TLR2 along with the NF-κB luciferase reporter. The cells were then stimulated with VZV-infected HELF or Pam3CSK4. Both VZV and Pam3CSK4 were able to activate NF-κB in the presence of either human or murine TLR2 (Fig. 2D). Thus, human cells expressing murine TLR2 are activated by VZV while murine cells expressing human TLR2 are not, suggesting that a human-specific cofactor or adapter molecule is required.

**Inhibition of IL-6 secretion in macrophages by siRNA targeting TLR2.** In order to prove that TLR2 participates in the activation of NF-κB in response to cell-associated VZV in vitro, we used siRNA that targeted human TLR2 in human monocyte-derived macrophages. A 21-nucleotide sequence targeting TLR2 from positions 75 to 90 in relation to the start codon (TLR2 siRNA) was introduced into human macrophages by lipofection. siRNA targeting TLR8 from positions 416 to 434 (TLR8 siRNA) was used as a non-TLR2 siRNA control in parallel tests for transfection-associated cell toxicity. Transfection of TLR2 siRNA resulted in a maximal decrease of surface TLR2 antibody staining 48 h after transfection as measured by flow cytometry, shown in Fig. 3.

In these experiments, monocyte-derived macrophages were transfected with TLR2 siRNA (or TLR8 siRNA as a control). Forty-eight hours later, cells were stained with APC-conjugated antibody against TLR2. Cells transfected with TLR2 siRNA (Fig. 3, upper panel, shaded histogram) had a lesser degree of TLR2 staining than cells transfected with control TLR8 siRNA (Fig. 3, upper panel, thin-lined histogram). TLR2 siRNA-transfected cells or control siRNA-transfected cells were each stained with isotype control antibody (Fig. 3, lower panel, bold-lined and thin-lined histograms, respectively). Of note, cells transfected with control TLR8 siRNA had full expression of TLR2 at the cell surface compared to untransfected cells (data not shown).

We then measured IL-6 production by macrophages in response to either VZV-infected HELF or the TLR2 ligand Pam3CSK4 in human macrophages transfected with TLR2 siRNA or control siRNA. Based on flow cytometry data, we predicted that the maximal knockdown of TLR2 by siRNA occurs 48 h after transfection, so at this point, macrophages were stimulated with either VZV or Pam3CSK4 (Fig. 4). As a control, some cells were transfected with siRNA targeting TLR8 and stimulated with the TLR7/TLR8 ligand R-848. Supernatants were collected after 8 h, and IL-6 was measured by ELISA. Cells with TLR2 knockdown had a 30 to 40% decrease in IL-6 responses to the TLR2 agonists VZV-infected HELF and Pam3CSK4 compared to that of control cells with TLR8 knockdown. Accordingly, TLR8 siRNA transfection caused a 50% reduction in IL-6 in response to R-848 (P < 0.01) but not in response to VZV-infected HELF or Pam3CSK4. As human TLR8 has been shown to interact only with single-stranded RNA viruses (6) and not with double-stranded DNA viruses, such as the herpesviruses, cytokine induction by VZV is not expected to be dependent on activation of TLR8. Cells transfected with TLR2 siRNA (or control siRNA) were stimulated with LPS to ensure that nonspecific suppression from a particular siRNA did not account for differences in cytokine production. All cells had a robust IL-6 response to LPS, which is mediated through TLR4. Conditions were performed in triplicate, and the siRNA experiments were
DISCUSSION

Using HEK293 cells as a model, we demonstrated that VZV induced NF-κB activity when TLR2 and CD14 were expressed. We then examined cytokine production in response to VZV in human monocytes, a natural host cell for VZV, and found significant induction of IL-6 and IL-8. We discovered that murine cells expressing TLR2, of either human or murine origin, did not produce cytokines in response to VZV, although HEK293 cells expressing murine TLR2 were responsive to VZV. Finally, partial silencing of TLR2 expression in primary human macrophages by RNA interference decreased the ability of these cells to secrete IL-6 after activation with VZV.

Our finding that TLR2 activates inflammation in human cells infected by VZV mirrors its role in infection by HSV, a closely related virus, and by cytomegalovirus, a beta-herpesvirus. TLR2 may participate in the host inflammatory response to these viruses, which ranges from mild to severe. We also found that various murine cells do not activate NF-κB in response to this virus, even when human TLR2 is expressed in murine cells in vitro. VZV has a narrow host range restricted to humans and selected cell types of primate and guinea pig origin; mice and cells of murine origin are not susceptible to VZV infection (22). In contrast, HSV has a broader host range and activates both human and murine cells in a TLR2-dependent manner. However, we demonstrated that activation of NF-κB by VZV can occur in human cells expressing murine TLR2 sequences. Human coreceptors, including those involved in viral entry, may play a role in TLR2 activation and induction of inflammatory cytokines and thereby explain the species specificity of IL-6 induction by VZV. Our data suggest that human CD14 may participate in VZV signaling, as CD14 enhanced the activation of NF-κB in HEK/TLR2 cells. At present, we do not know which component of VZV activates TLR2, except that TLR2-mediated induction of NF-κB does not appear to require live virus or viral replication. However, immediate-early and early events following infection could play a role. In ongoing studies, we are examining viral envelope components to determine which can specifically act as a TLR2 ligand.

To demonstrate that TLR2 participates in the induction of human inflammatory cytokines by VZV, we successfully established a model that utilizes RNA interference in primary human cells. We achieved a functional knockdown of cellular responses to the TLR2 ligand VZV and Pam3CSK4 of 30 to 40%. The similarity in degree to which IL-6 production was reduced suggests that a large amount of the inflammatory cytokine response by macrophages to cell-associated VZV is due to its TLR2-activating properties. siRNAs have potentially broad applications in the study of pathogens and TLRs. For example, the role of TLRs may be established for microbial pathogens that specifically trigger inflammation in human cells. Furthermore, siRNAs against TLR8 can be used to characterize the role of TLR8 in cytokine induction by single-stranded RNA viruses, which appear to be the natural ligand for TLR8 (6). This is advantageous since animal studies of TLR8 have been lacking, as murine TLR8 appears to be nonfunctional (7, 9).

In summary, our results demonstrate that VZV induces cellular activation of inflammatory pathways in a species-specific, TLR2-dependent manner. These results suggest that, as with HSV, TLR2 participates in the early immune response after VZV infection and contributes to the clinical presentation of symptomatic primary or reactivation disease with VZV, char-
characterized by fever and/or rash (15). This innate host immune response may be linked with specific cell-mediated immune responses that participate in severe complications of VZV infection, including thrombocytopenia, encephalitis, hepatitis, and pneumonitis. Such clinical findings are often associated with the production of inflammatory cytokines that may be linked with host TLR2 recognition of herpesviruses.

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