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Varicella Zoster Virus Downregulates Expression of the Nonclassical Antigen Presentation Molecule CD1d

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Background. The nonclassical antigen presentation molecule CD1d presents lipid antigens to invariant natural killer T (iNKT) cells. Activation of these cells triggers a rapid cytokine response providing an interface between innate and adaptive immune responses. The importance of CD1d and iNKT cells in varicella zoster virus (VZV) infection has been emphasized by clinical reports of individuals with CD1d or iNKT cell deficiencies experiencing severe, disseminated varicella postvaccination.

Methods. Three strains of VZV (VZV-S, rOka, and VZV rOka-66S) were used to infect Jurkat cells. Flow cytometry of VZV- and mock-infected cells assessed the modulatory impact of VZV on CD1d protein. Infected cell supernatant and transwell co-culture experiments explored the role of soluble factors in VZV-mediated immunomodulation. CD1d transcripts were assessed by reverse-transcription polymerase chain reaction.

Results. Surface and intracellular flow cytometry demonstrated that CD1d was strikingly downregulated by VZV-S and rOka in both infected and VZV antigen-negative cells compared to mock. CD1d downregulation is cell-contact dependent and CD1d transcripts are targeted by VZV. Mechanistic investigations using rOka-66S (unable to express the viral kinase ORF66) implicate this protein in CD1d modulation in infected cells.

Conclusions. VZV implements multiple mechanisms targeting both CD1d transcript and protein. This provides evidence of VZV interaction with and manipulation of the CD1d–iNKT cell axis.

Keywords. varicella zoster virus; VZV; immune modulation; CD1d.

Varicella zoster virus (VZV) is a ubiquitous human pathogen capable of establishing lifelong latency within the sensory ganglia. VZV is responsible for 2 distinct diseases: varicella (chickenpox) at primary infection, and herpes zoster (shingles) following viral reactivation from latency. The ability to establish latency is a common feature among herpesviruses and involves multiple viral immune system evasion strategies.

VZV is highly T-cell tropic, and these cells are critical for the spread of VZV from the primary site of infection, the respiratory mucosa, throughout the circulation, and to the skin where infection results in the formation of cutaneous lesions [1, 2]. As major histocompatibility complex class I (MHC-I) and class II (MHC-II) presentation of peptide antigens has a critical role in T-cell pathogen detection, it is unsurprising that VZV actively downregulates cell-surface expression of these molecules. VZV targets MHC-I as it traffics from the Golgi to the cell surface [3], with the VZV-encoded serine/threonine kinase at least partially responsible for the downregulation [4]. VZV also targets the interferon gamma (IFN-γ) signaling pathway, which blocks IFN-γ–induced cell-surface MHC-II in fibroblasts [5]. Targeting of these pathways reduces T-cell recognition of VZV, allowing the virus to replicate and spread efficiently.

VZV immune evasion is not limited to classical T cells, with mucosal-associated invariant T (MAIT) cells also targeted. Recently we demonstrated that VZV downregulates cell-surface expression of MHC-I–related protein (MR1), which presents bacterial riboflavin metabolite ligands to MAIT cells [6]. These T cells, along with invariant natural killer T (iNKT) cells are nonconventional "innate-like" αβ T cells that express cell-surface markers characteristic of natural killer (NK) cells, have the capacity to react in a proinflammatory manner without differentiation, and respond to inflammatory cytokines in the absence of T-cell receptor signaling [7–9]. iNKT cells are restricted to lipid antigens presented on MHC-I–like molecule CD1d. Although MAIT and iNKT cells respond to antigens of nonviral origin, both have been targeted by viruses, particularly human herpesviruses [10–15]. Most stages in the CD1d biosynthesis pathway have proven vulnerable to herpesvirus immune evasion, ultimately impairing iNKT cell function [10–12, 15–17].

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Clinical reports highlight the association between CD1d and iNKT cell deficiencies and VZV disease severity following vaccination with the live-attenuated varicella vaccine in children [18, 19]. A decreased iNKT cell count has also been linked to patients with multiple VZV reactivations, suggesting inability of the immune system to control VZV [20]. Remarkably, despite the body of evidence identifying a relationship between the CD1d–iNKT cell axis and VZV, there has been no investigation on how VZV may manipulate this subset of the immune response. Here, we demonstrate the direct involvement of VZV infection in the downregulation of cell-surface and total CD1d expression. We identify that multiple VZV strains (VZV-S and rOka) can downmodulate CD1d expression and identify VZV ORF66 as partially mediating CD1d downregulation. This study illuminates the influence VZV has on the innate-like adaptive immune response, enhancing our understanding of VZV pathogenesis and immune modulatory potential.

METHODS

Cells

ARPE-19 human epithelial cells were maintained in Dulbecco's modified Eagle medium with high glucose and sodium pyruvate (Gibco) supplemented with 10% fetal calf serum (FCS; Serana), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). The Jurkat human T-lymphocyte cell line was maintained in RPMI medium (RPMI 1640 with L-glutamine, Gibco) supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Viruses and Co-culture

Laboratory-adapted, virulent VZV-S [21, 22], recombinant VZV Oka strain (rOka), and rOka-66S, a recombinant strain unable to express ORF66, were propagated and maintained in ARPE-19 cells (viruses provided by Professor Arvin, Stanford University). Both rOka and rOka-66S are cosmid-derived viruses generated from the parental Oka strain (GenBank accession number AB097933.1) [23]. VZV-infected ARPE-19 cells demonstrating cytopathic effect in 65%–90% of cells (inoculum) were CellTrace violet (CTV; Invitrogen) labeled as described previously [24] before being added to Jurkat cells at a 1:1 ratio in supplemented RPMI. In transwell experiments, inoculum was plated in the wells as usual and Jurkat cells were co-cultured 1:1 within or without 0.4 µm pore polycarbonate membrane inserts (Corning).

Flow Cytometry

Cells were stained with LiveDead fixable blue dead cell stain (Invitrogen) as per manufacturer's instructions. Cells were washed and stained with primary antibodies in FACS buffer (phosphate-buffered saline with 1% FCS and 10 mM ethylenediaminetetraacetic acid) for 40 minutes at 4°C. Cell surface–stained samples were fixed with BD Cytofix buffer (BD Biosciences). Monoclonal antibodies utilized included anti-human CD1d-PE (51.1; Invitrogen), anti-VZV gE:gI-Dy488 (SG1, Meridian Life Sciences), anti-human MHC-I-APC (G46–2.6; BD Biosciences), and anti-human CD71-BV650 (CY1G4; Biolegend). Matched isotype control antibodies were utilized. For intracellular staining, cells were fixed and permeabilized with BD Cytofix/Perm solution, washed, and permeabilization maintained with BD Perm/Wash buffer (BD Biosciences). Sample data were acquired using a BD LSRII cytometer and analyzed with FlowJo software version 10.7.2 (FlowJo, Ashland, Oregon). Cell populations were gated based on forward and side scatter. Singlet cells were gated as CTV-negative, to exclude inoculum, and viability dye-negative, to select for live cells. Cells positive for the VZV gE:gI antigen were deemed infected, with gE:gI-negative cells defined antigen-negative (Supplementary Figure 1). Graphs were generated and statistical significance was determined by 2-way analysis of variance (ANOVA) with Tukey multiple comparisons test using GraphPad Prism software version 9.

Reverse-Transcription Quantitative Polymerase Chain Reaction

RNA was extracted using the Bioline RNA II Isolate kit as per manufacturer's instructions from inoculum only, Jurkat cells removed from co-culture, or Jurkat cells alone. RNA concentration was standardized between each biological repeat for complementary DNA (cDNA) synthesis by reverse-transcription polymerase chain reaction (RT-qPCR). Quantitative PCR was performed in duplicate on a QuantStudio 7 Pro Real-Time PCR system (Applied Biosciences) using PowerTrack SYBR Green mix (Applied Biosciences), as per the manufacturer's instructions. Primers were as follows: CD1d: 5′-AGCTACACC TCCATGGGCTT-3′ and 5′-GGTGTGATGAAAGCTGCCT C-3′; 18S: 5′-GTAACCCGTTGAACCCCATT-3′ and 5′-CCA TCCAATCGGTAGTAGCG-3′. For each primer set, a negative control (water) and positive control dilution standards were run to validate primer efficiency. The cycle threshold (Ct) value was used to calculate the ΔCt value of CD1d, normalizing to the 18S housekeeping gene. Fold change of CD1d transcripts between samples, relative to Jurkat cells grown in isolation, was calculated using $2^{-(\Delta\Delta Ct)}$. Statistical significance was determined by 1-way ANOVA with Tukey multiple comparison test.

RESULTS

CD1d Is Downregulated in VZV-Infected Jurkat Cells

Given VZV downregulates classical and nonclassical antigen presentation molecules, and the body of clinical evidence to suggest the CD1d–iNKT cell axis is involved in VZV control, we investigated if VZV also targeted CD1d. The Jurkat human T-lymphocyte line was selected due to high cell-surface expression of CD1d [25]. Due to the well-documented permissiveness of T cells to VZV infection [26], it was expected that Jurkat cells

would be permissive, despite not finding published studies showing in vitro VZV infection of Jurkat cells. As VZV is highly cell-associated in vitro [27], a cell-associated infection model was used, where inoculum cells, consisting of CTV-labeled VZV-S–infected ARPE-19 cells, were co-cultured with Jurkat cells before flow cytometry staining. With the asynchronous infection associated with cell-associated infection and 1 round of VZV replication expected to take 9–12 hours [28], co-culture times of 24 and 48 hours were selected. Following exclusion of the inoculum and nonviable cells, Jurkat cells were gated based on presence or absence of the VZV gE:gI antigen. The gE:gI heterodimer is expressed on the cell-surface of infected cells and is a marker of late VZV infection [29]. Mock-infected Jurkat cells demonstrated no gE:gI staining. Jurkat cells were highly permissive to VZV-S infection with detectable gE:gI at 24 hours postinfection (hpi) (25.3% ± 5.6%) and 48 hpi (32.7% ± 4.6%) (data not shown). Cell-surface and intracellular flow cytometry antibody staining of the Jurkat cells was used to analyze CD1d cell-surface and total expression, respectively. MHC-I downregulation by VZV is well established [3, 4, 30], whereas the transferrin receptor, CD71, has been shown to be unaffected by VZV infection [3, 5]; therefore, these molecules were analyzed to validate VZV-mediated cell-surface downregulation and as a negative control, respectively.

At 24 and 48 hpi, cell-surface and total CD1d was significantly downregulated in VZV-infected (gE:gI positive) cells relative to mock ($P < .0001$; Figure 1*A* and 1*B*). A greater fold downregulation in CD1d was observed at 48 hpi. MHC-I was downregulated in VZV-infected cells at 24 hpi ($P < .05$) and 48 hpi ($P < .0001$) compared to mock (Figure 1*C*). CD71 was not significantly modulated at 24 hpi, but at 48 hpi was significantly downregulated in VZV-infected cells ($P < .01$; Figure 1*D*). These findings indicate that VZV infection can downregulate cell-surface and total CD1d.

VZV rOka Also Downregulates CD1d, and ORF66 Partially Contributes

Having observed that VZV-S downregulates CD1d at the cell-surface and total protein level, we sought to investigate if this phenomenon was virus strain specific and began mechanistic investigations. Given the clinical reports detailing CD1d- and iNKT cell–deficient patients experiencing disseminated varicella following vaccination [18, 19], we assessed the effect the recombinant vaccine strain, rOka, has on CD1d expression. Additionally, the potential role of VZV ORF66 was investigated due to the viral protein's partial involvement in MHC-I downregulation, and the involvement of herpes simplex virus type 1 (HSV-1) homologue of ORF66, US3, in CD1d downregulation [4, 14]. The involvement of ORF66 was investigated using a rOka virus unable to express ORF66 due to insertion of multiple stop codons [23]. Modulation of cell-surface CD1d, MHC-I, and CD71, in addition to total CD1d, was assessed in VZV-infected Jurkat cells as performed previously.

The VZV rOka strain showed reduced infection compared to VZV-S, with detectable gE:gI in 8.4% (±1.3%) at 24 hpi and 9.6% (±0.5%) at 48 hpi. Similarly, rOka-66S–infected cells made up 16.8% (±2.9%) at 24 hpi and 13.8% (±3.0%) at 48 hpi (data not shown). The rOka strain strongly downregulated cell-surface CD1d in gE:gI-positive cells at 24 and 48 hpi, compared to mock-infected cells ($P < .0001$; Figure 2*A*). As observed for VZV-S, CD1d downregulation was more profound at 48 hpi. Total CD1d followed an almost identical trend with significant loss of CD1d compared to mock at both timepoints ($P < .0001$; Figure 2*B*). Cell-surface MHC-I was not downregulated at 24 hpi when infected with rOka, but was decreased by 48 hpi ($P < .0001$) compared to mock (Figure 2*C*). No modulation of cell-surface CD71 was observed in VZV-infected cells compared to mock-infected cells (Figure 2*D*).

At 24 hpi, rOka-66S–infected cells showed moderate loss of cell-surface CD1d relative to mock ($P < .01$), whereas by 48 hpi downregulation from the cell-surface was more pronounced ($P < .001$) (Figure 2*A*). Similar trends were observed for total CD1d with significant downregulation at 24 hpi ($P < .001$) and 48 hpi ($P < .0001$) in rOka-66S–infected cells, compared to mock (Figure 2*B*). Dissimilar to VZV-S and rOka, there was no significant loss of cell-surface MHC-I expression at either timepoint in rOka-66S–infected cells (Figure 2*C*). CD71 cell-surface expression was upregulated at both timepoints compared to mock ($P < .05$ and $P < .001$, respectively; Figure 2*D*).

In assessing the difference between rOka- and rOka-66S–infected cells, the loss of CD1d from the cell surface is more pronounced at 24 hpi ($P < .0001$) and 48 hpi ($P < .01$) (Figure 2*A*), as is total CD1d ($P < .01$ and $P < .001$, respectively; Figure 2*B*). As expected, MHC-I is also significantly more downregulated in rOka-infected cells than rOka-66S–infected cells at both timepoints ($P < .001$ and $P < .0001$; Figure 2*C*). Contrastingly, rOka-66S–infected cells display significantly higher levels of cell-surface CD71 than rOka-infected cells ($P < .05$ and $P < 001$; Figure 2*D*). These results demonstrate that CD1d downregulation is not VZV-S specific and is also observed for the rOka strain. In the context of infection, ORF66 plays a partial role in CD1d downregulation.

CD1d Is Downregulated in VZV-Exposed Antigen-Negative Cells

As previously outlined, viable Jurkat cells were gated based on the detection of VZV gE:gI. Due to the asynchronous infection of the Jurkat cells, 74.7% (±9.6%) to 91.5% (±2.2%) of Jurkat cells were antigen negative at 24 hpi, and 67.3% (±8%) to 90.4% (±0.9%) at 48 hpi (data not shown). Despite infected cells being the focus, due to the large proportion of gE:gI-negative cells, we also analyzed these cells as previously described.

Interestingly, CD1d is profoundly downregulated in gE:gI-negative cells at the cell surface and total protein level for

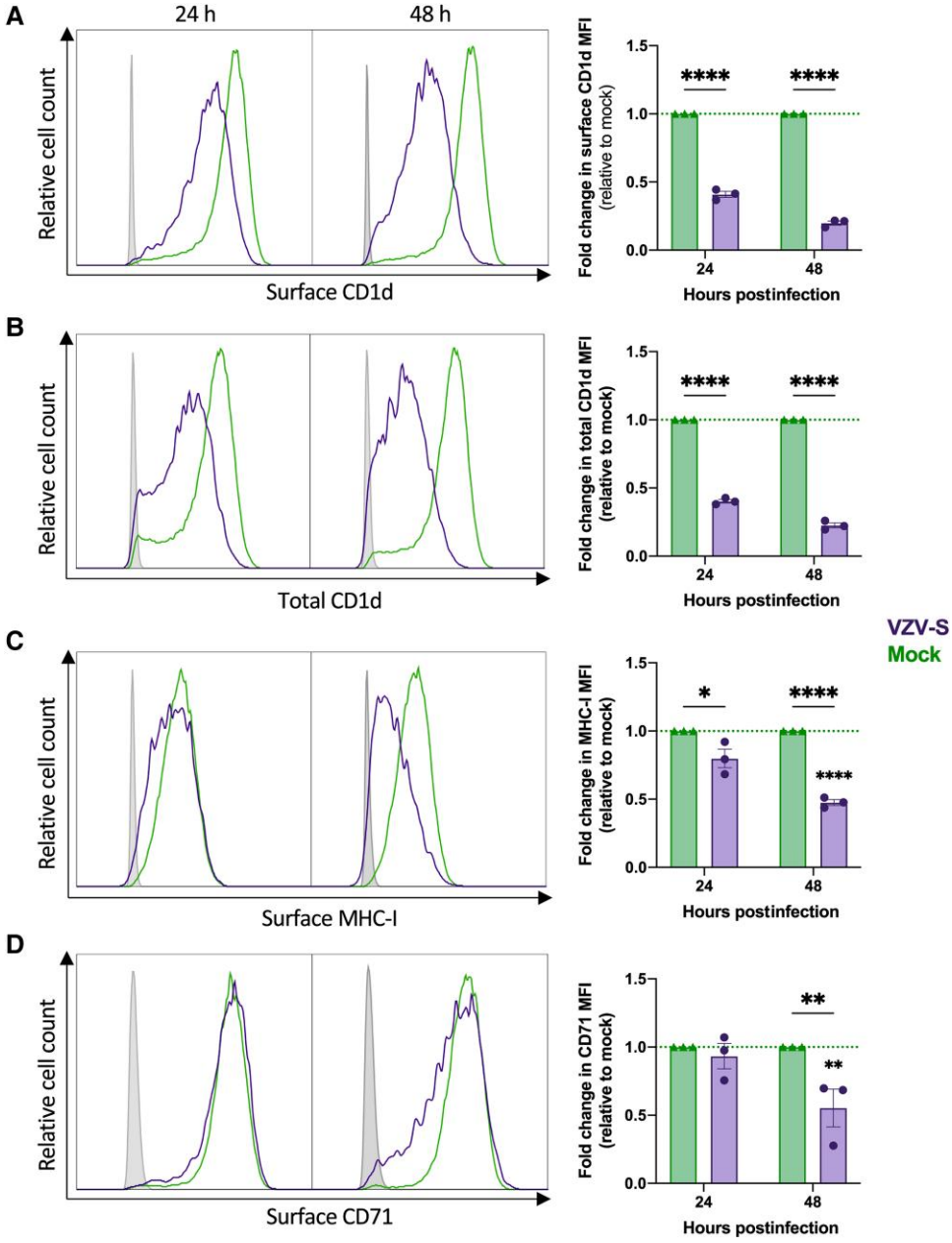

Figure 1. Varicella zoster virus (VZV) downregulates CD1d in Jurkat cells at the cell-surface and total cell level. Inoculum ARPE-19 cells were infected with VZV-S until 50%–75% of cells were showing cytopathic effects or left uninfected. The inoculums were labeled and co-cultured with Jurkat cells at a 1:1 ratio. At 24 and 48 h postinfection, cells were harvested and stained with viability dye and fluorescent-conjugated primary antibodies for flow cytometry analysis. Representative flow cytometry histograms of surface CD1d (*A*), total CD1d (*B*), surface major histocompatibility complex class I (MHC-I; *C*) and surface CD71 (*D*) in VZV-S–infected (purple) and mock-infected (green) Jurkat cells compared to isotype control-stained (gray). Graphs depict mean (± standard error of the mean) relative mean fluorescence intensity (MFI) fold change relative to mock-infected cells (green and dotted line) for the same antigen as shown in the histogram (n = 3 biological repeats). Statistical significance was determined by 2-way analysis of variance with Tukey multiple comparisons test. $^{*}P < .05$, $^{**}P > .01$, $^{****}P < .0001$.

all analyzed VZV strains at 24 and 48 hpi compared to mock (Figure 3*A* and 3*B*). VZV-S and rOka significantly decreased cell-surface CD1d at 24 hpi ($P < .001$), as did rOka-66S ($P < .01$). CD1d surface expression was further decreased at 48 hpi for all strains ($P < .0001$). Total CD1d was significantly downregulated at both timepoints for all strains ($P < .0001$). VZV rOka was slightly less potent at downregulating total CD1d compared to VZV-S at 24 hpi ($P < .05$), and both rOka and rOka-66S were at 48 hpi ($P < .05$). Unlike in the gE:gI-positive cells, there is no difference in the downregulation of CD1d on cells exposed to rOka and rOka-66S. There was no loss of cell-surface MHC-I in the antigen-negative cells

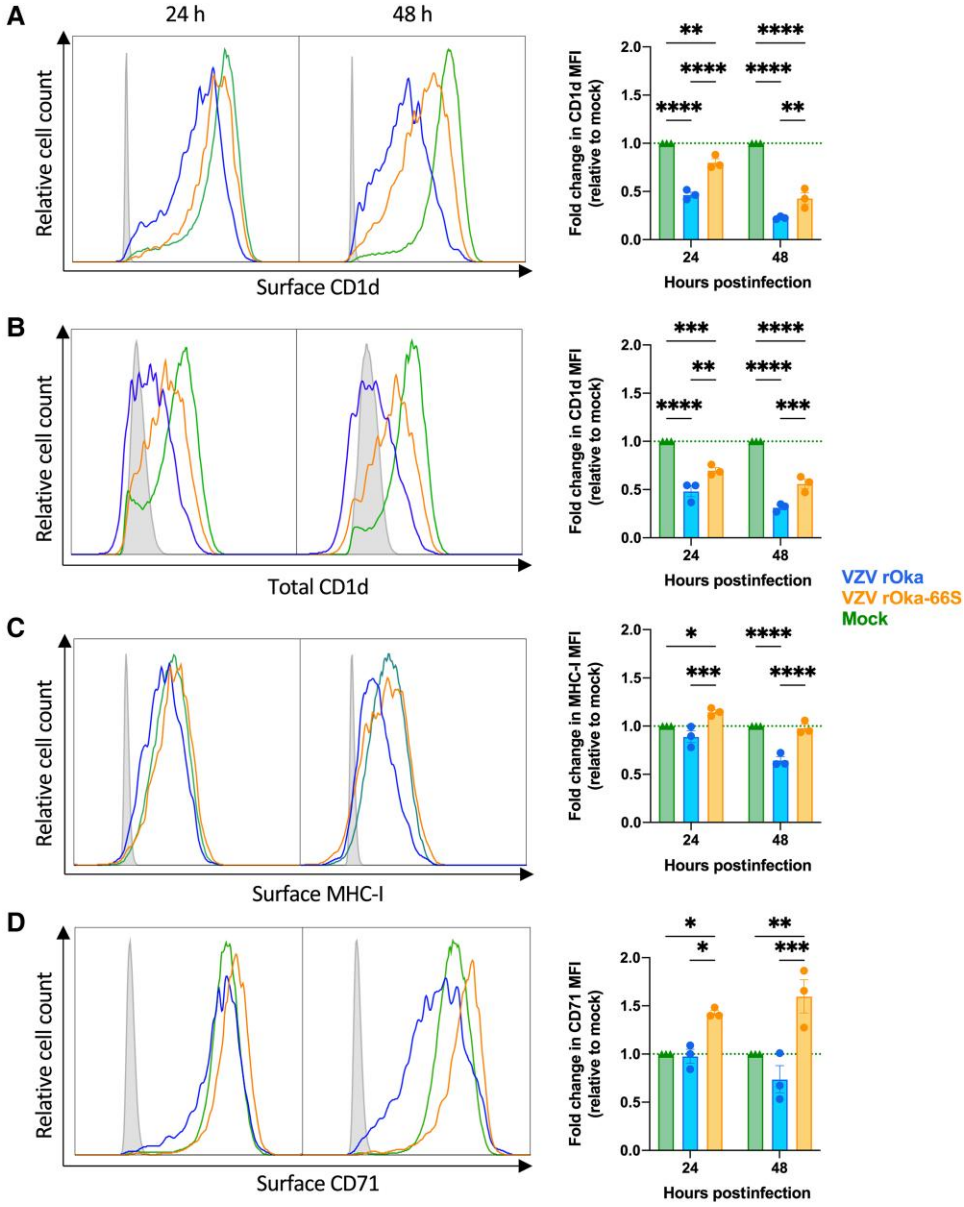

Figure 2. Varicella zoster virus (VZV) rOka and rOka-66S downregulate CD1d in Jurkat cells at the cell-surface and total cell level. Inoculum ARPE-19 cells were infected with the appropriate VZV strain until 50%–75% of cells were showing cytopathic effects or mock-infected. The inoculums were labeled and co-cultured with the Jurkat cells at a 1:1 ratio. At 24 and 48 h postinfection, cells were harvested and stained with viability dye and fluorescent-conjugated primary antibodies for flow cytometry analysis. Representative flow cytometry histograms of surface CD1d (*A*), total CD1d (*B*), surface major histocompatibility complex class I (MHC-I; *C*), and surface CD71 (*D*) in VZV rOka-infected (blue), VZV rOka-66S–infected (orange), and mock-infected (green) Jurkat cells compared to isotype control-stained (gray). Graphs depict mean (± standard error of the mean) relative mean fluorescence intensity (MFI) fold change relative to mock-infected cells (green and dotted line) for the same antigen as shown in the histogram (n = 3 biological repeats). Statistical significance was determined by 2-way analysis of variance with Tukey multiple comparisons test. $^{*}P < .05$, $^{**}P > .01$, $^{***}P < .001$, $^{****}P < .0001$.

exposed to any strain compared to mock (Figure 3*C*). A slight upregulation of MHC-I was observed on these cells. A similar pattern was observed for cell-surface CD71, with no loss of expression on gE:gI-negative cells compared to mock, with upregulation observed for rOka-exposed cells at the later timepoint ($P < .01$; Figure 3*D*). This analysis indicates that downregulation of cell-surface and total CD1d in both VZV-infected and antigen-negative Jurkat cells is not observed for MHC-I and CD71.

VZV-Mediated CD1d Downregulation Is Contact Dependent

The striking loss of CD1d in the antigen-negative cells was surprising and prompted further investigation. Herpesviruses are known to express secreted factors for immune evasion [31, 32],

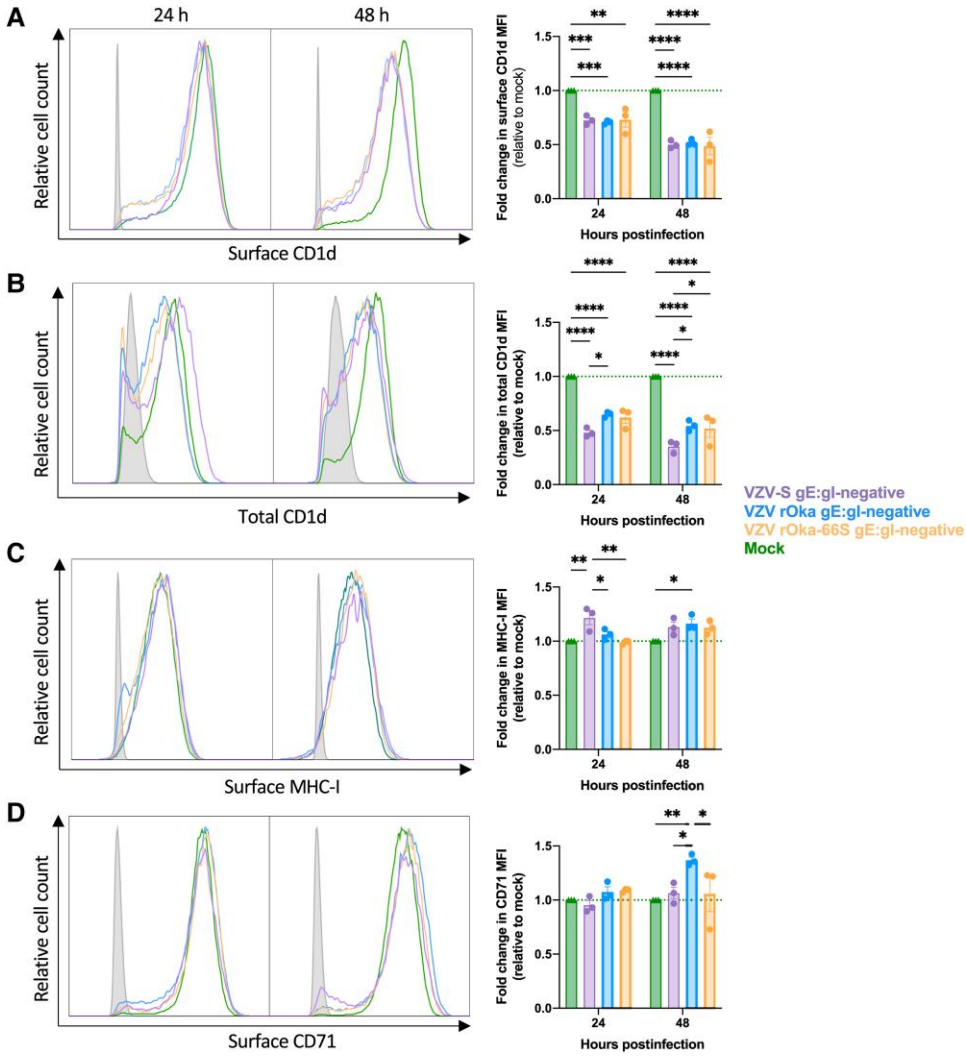

Figure 3. CD1d is downregulated in varicella zoster virus (VZV) antigen-negative cells. Inoculum ARPE-19 cells were infected with the appropriate VZV strain until 50%–75% of cells were showing cytopathic effects or mock-infected. The inoculums were labeled and co-cultured with the Jurkat cells at a 1:1 ratio. At 24 and 48 h postinfection, cells were harvested and stained with viability dye and fluorescent-conjugated primary antibodies for flow cytometry analysis. Representative flow cytometry histograms of surface CD1d (*A*), total CD1d (*B*), surface major histocompatibility complex class I (MHC-I; *C*), and surface CD71 (*D*) in VZV-S– (purple), VZV rOka- (blue), VZV rOka-66S– (orange), and mock- (green) exposed, gE:gI antigen–negative Jurkat cells compared to isotype control-stained (gray). Graphs depict mean (± standard error of the mean) relative mean fluorescence intensity (MFI) fold change relative to mock-infected cells (green and dotted line) for the same antigen as shown in the histogram (n = 3 biological repeats). Statistical significance was determined by 2-way analysis of variance with Tukey multiple comparisons test. $^{*}P < .05$, $^{**}P > .01$, $^{***}P < .001$, $^{****}P < .0001$.

so we sought to determine whether a secreted factor from the infected cells, either the inoculum or target, was contributing to the profound downregulation of CD1d on the antigen-negative cells. Supernatants collected from VZV- and mock-infected inoculum–Jurkat cell co-cultures were diluted in fresh media (1:1 or 1:2) and added to fresh Jurkat cells, harvesting after 24 hours for flow cytometry analysis as previously described. There was no detection of VZV gE:gI on Jurkat cells following culture with VZV-infected cell supernatant (data not shown), indicating an absence of infectious virions, as expected [27].

There was no downregulation of cell-surface CD1d on Jurkat cells cultured with supernatant harvested from VZV-infected cells, compared to supernatant from mock cultures, at either dilution (1:1 or 1:2) (Figure 4*A*). There was a slight upregulation ($P < .05$) in cells exposed to 1:1 diluted supernatant from 48 hpi. In previous experiments MHC-I was not downregulated in antigen-negative cells; this was further supported by the lack of modulation, or upregulation in cells exposed to diluted supernatant (Figure 4*B*).

To assess the requirement for cell-to-cell contact with an infected cell to downregulate CD1d, transwells were used to separate the Jurkat cells from the VZV-infected or mock inoculum for 24 and 48 hours before analysis by flow cytometry. Nonseparated cultures were included as a control. No gE:gI was detected in Jurkat cells separated from the VZV- and mock-infected inoculum, confirming the lack of virions within the culture media (data not shown).

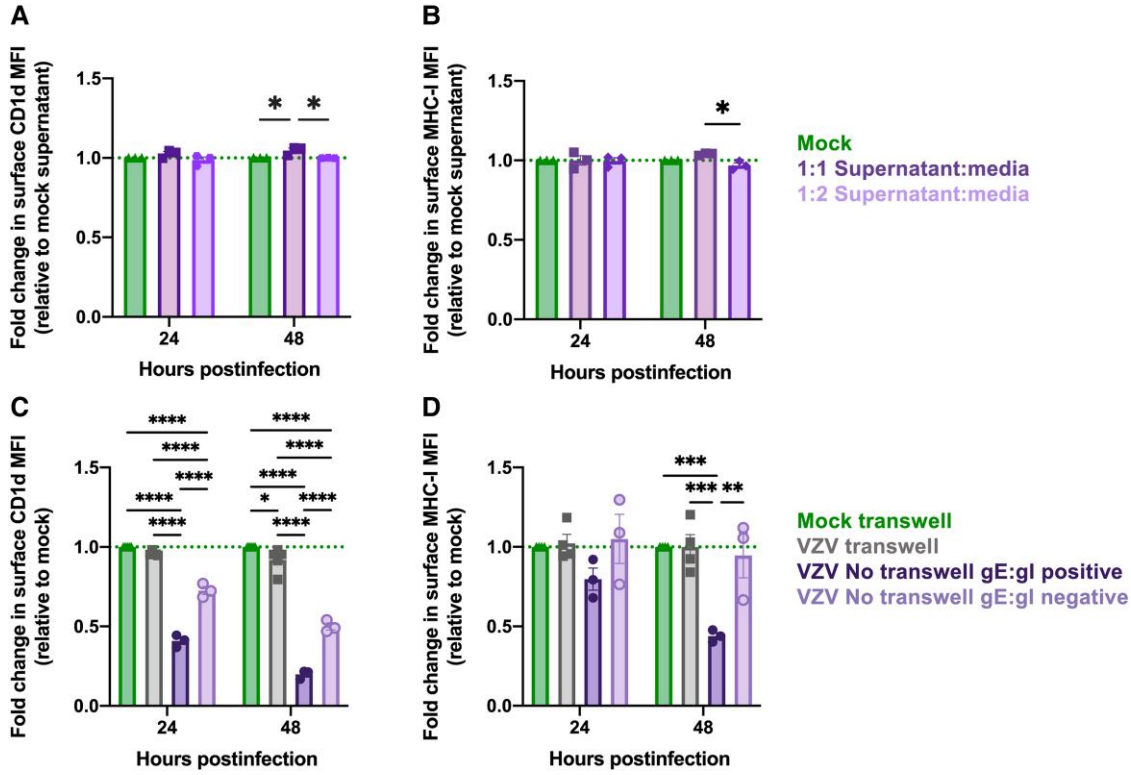

Figure 4. Cell-to-cell contact required for CD1d downregulation on Jurkat cells; supernatant from infected cells alone is not sufficient. *A* and *B*, Inoculum ARPE-19 cells were infected with varicella zoster virus (VZV)–S until 50%–75% of cells were showing cytopathic effects or mock-infected. Supernatants were collected and clarified at 24 and 48 h postinfection. The supernatants were applied to fresh, uninfected Jurkat cells for 24 h before harvesting and staining for flow cytometry analysis. Graphs depict mean (± standard error of the mean [SEM]) relative mean fluorescence intensity (MFI) fold change relative to mock-infected supernatant (green and dotted line) for cell-surface CD1d (*A*) and major histocompatibility complex class I (MHC-I; *B*) expression in Jurkat cells exposed to supernatant-diluted 1:1 (dark purple) or 1:2 (light purple) in RPMI media (n = 3 biological repeats). *C* and *D*, Jurkat cells were separated from mock- and VZV-infected ARPE-19 inoculum cells by 0.4 µm transwell membranes or co-cultured together as previously. At 24 and 48 h postinfection, Jurkat cells were harvested and stained for flow cytometry analysis. Graphs depict mean (±SEM) relative MFI fold change relative to no transwell mock-infected Jurkat cells (green and dotted line) for cell-surface CD1d (*C*) and surface MHC-I (*D*) in VZV-infected transwell-cultured (gray) and co-cultured VZV-S gE:gI-positive (purple) Jurkat cells (n = 3 biological repeats). For both experiments, statistical significance was determined by 2-way analysis of variance with Tukey multiple comparisons test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

As previously shown, gE:gI-positive and -negative populations were identified within the co-cultured samples. Cell-surface CD1d levels on Jurkat cells separated from the VZV inoculum were equivalent with those observed on mock transwell-cultured cells at 24 and 48 hpi (Figure 4*C*). As expected, no transwell-cultured cells reproduced earlier results for CD1d ($P < .0001$; Figure 4*C*). For MHC-I, significance was only observed at 48 hpi, with loss of cell-surface MHC-I in the no transwell, co-cultured gE:gI-positive cells compared to mock cells ($P < .001$), VZV transwell-cultured cells ($P < .001$) and gE:gI-negative cells ($P < .01$) (Figure 4*D*), reproducing previous results. These results highlight the requirement of cell-to-cell contact with VZV-infected cells to reduce cell-surface CD1d.

VZV Targets CD1d Transcripts

Alphaherpesviruses are known to shut down host cell transcription during replication. VZV decreases messenger RNA transcript abundance of a number of cellular proteins including MHC-II [5]. Due to the profound loss of total CD1d protein, we sought to investigate if VZV was targeting transcripts using RT-qPCR. Jurkat cells were cultured with mock- or VZV-infected inoculum for 24 hours before RNA was extracted for cDNA synthesis and then qPCR was performed. Housekeeping genes including 18S, GAPDH, and PPIA were assessed for stability between the mock- and VZV-infected samples (data not shown). 18S remained stable and was used for normalization.

CD1d transcript abundance was profoundly decreased in VZV-exposed Jurkat cells compared to mock-exposed Jurkat cells and Jurkat cells alone ($P < .001$; Figure 5). CD1d transcripts remained unchanged between mock-exposed Jurkat cells and those grown in the absence of inoculum. These data suggest that VZV is targeting CD1d at the transcript level, likely contributing to the loss of surface and total CD1d observed.

DISCUSSION

Downregulation of CD1d is a mechanism implemented by many viruses including human immunodeficiency virus, severe

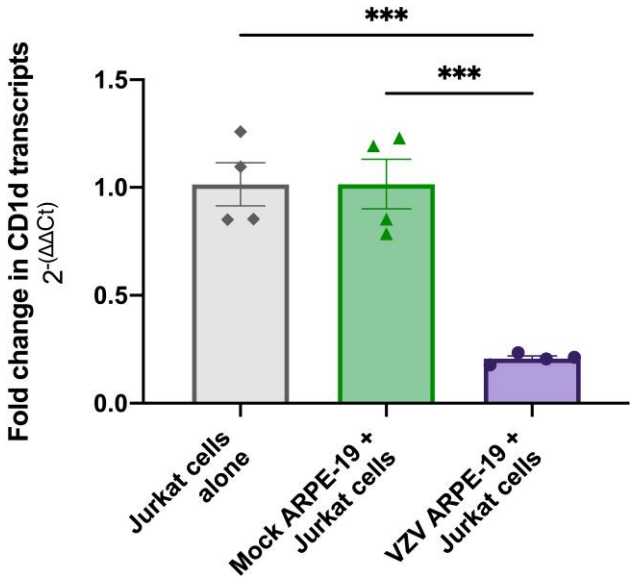

Figure 5. CD1d transcripts are downregulated in Jurkat cells exposed to varicella zoster virus (VZV)–infected inoculum. Inoculum ARPE-19 cells were infected with VZV-S until 50%–75% of cells were showing cytopathic effects or mock-infected. Jurkat cells were added to the inoculum or grown alone. At 24 h postinfection Jurkat cells were removed from the inoculum by pipetting and RNA was extracted. Complementary DNA was synthesized from the standardized RNA concentration for quantitative polymerase chain reaction (qPCR). 18S transcripts were used for ΔCt CD1d transcript normalization. Graphs depict mean (± standard error of the mean) fold change in CD1d transcripts for mock co-cultured Jurkat cells (green) and VZV-infected co-cultured Jurkat cells (purple) as determined by $2^{-(\Delta\Delta Ct)}$ relative to Jurkat cells alone (gray). qPCR was performed in duplicate (n = 4 biological repeats). Statistical significance was determined by 1-way analysis of variance with Tukey multiple comparisons test. *** $P < .001$.

acute respiratory syndrome coronavirus 2, vaccinia virus, human papillomavirus, HSV-1, human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), and Kaposi sarcoma–associated herpesvirus [15, 33–38]. The different taxonomy of these viruses highlights the critical nature of the CD1d–iNKT cell axis in controlling viral infections. We demonstrated in this study that VZV downregulates CD1d in infected cells; however, unique to our study is the CD1d downregulation observed in VZV-exposed, antigen-negative cells. To our knowledge there is no demonstration of downregulation in exposed cells associated with other viruses. In fact, in a study using green fluorescent protein (GFP)–expressing HSV-1 there was upregulation in GFP-negative cells compared to GFP-positive infected cells [39]. We hypothesized that VZV may possess a unique mechanism to reduce cell-surface and total CD1d in these exposed cells. Secreted factors are an immune evasion mechanism implemented by other herpesviruses, as observed for EBV s-gp42, which inhibits MHC-II–restricted antigen presentation to T cells [32] and the HCMV viral interleukin 10 homologue and HCMV-induced transforming growth factor–β1, preventing dendritic cell maturation [31]. Through infected cell supernatant culture and transwell culture, we found that the involvement of a soluble factor did not modulate CD1d. In this respect, VZV-infected and antigen-negative NK cell cytolytic function impairment has been shown to be cell-contact dependent [40]. It is plausible that requirement for cell contact is a shared phenotype between closely related alphaherpesviruses as inhibition of iNKT cell activation by HSV-1–infected keratinocytes was also shown to be contact dependent, as virus-containing supernatants were not sufficient to alter such activation [10].

Detection of gE:gI antigen is highly successful at determining VZV-infected cells; however, it is important to identify that not all gE:gI-negative cells are uninfected. Antigen-negative cells may be in early stages of infection, not expressing detectable levels of gE:gI, or abortively infected, in that virus has entered the cell but unsuccessfully replicated. Given a soluble factor is not involved, it will be critical to differentiate abortively infected, exposed, and early stage–infected cells. The VZV serine/threonine kinase ORF66 is homologous, with HSV-1 protein US3, which has been implicated in downregulation of MHC-I, MR1, and CD1d [14, 39, 41, 42]. HSV-1 US3 phosphorylates cellular motor protein KIF3A, which results in accumulation of CD1d within the cell [14, 15, 39]. Additionally, HSV-1 glycoprotein B (gB) forms complexes with CD1d and prevents cell-surface trafficking; however, both US3 and gB are required for effective cell-surface CD1d downregulation [14]. Our study indicates that ORF66 may contribute partially to CD1d downregulation in infected Jurkat cells; however, further investigation is required to delineate the mechanism. In contrast to the retention of CD1d by HSV-1, total CD1d was significantly decreased in VZV-infected cells. Our results suggest that VZV may implement different mechanisms, targeting molecule biosynthesis by decreasing CD1d transcript and the partial involvement of ORF66 in cell-surface and total CD1d protein loss. The CD1d transcript loss is striking and is likely occurring in cells at all stages of infection, not just the infected, as detected during flow cytometry. Unfortunately, this can only be theorized as the cells were not differentiated based on gE:gI expression before assessing transcript. Though VZV-mediated transcript modulation has not been elucidated, ORF63, which encodes an immediate early protein (IE63) has been implicated [43]. This protein forms part of the VZV tegument and is in high abundance from early stages of infection [28], therefore could be acting on cells not expressing detectable gE:gI. Future work will endeavor to comprehensively delineate the different mechanisms implemented by VZV affecting both CD1d transcript and protein within infected and antigen-negative populations.

The loss of cell-surface CD71 was unexpected. This transferrin receptor has a rapid recycling pathway [44] and was downregulated in VZV-S–infected Jurkat cells but not rOka-infected cells or in gE:gI-negative cells. In VZV-infected human fibroblasts, CD71 remained unchanged [3], hence inclusion as a negative control. Mouse studies propose CD71 as a marker

for proliferating immature T cells and that downregulation was associated with ceased proliferation [45]. Viral replication can disrupt cell proliferation; therefore, CD71 loss may not be directly VZV-mediated, but rather a secondary effect. This theory supports the upregulation observed for rOka-66S–infected cells, as ORF66 is required for efficient replication within T cells [46], and cell proliferation may be less impacted than with VZV-S.

This study highlights that VZV targets the CD1d–iNKT cell axis through downregulation of CD1d. iNKT cell activation during viral infection is strongly reliant on CD1d antigen presentation [47], and iNKT cell functional impairment has been demonstrated in vitro for many herpesviruses [10–12, 14]. Following our current observations, assessment of iNKT cell functional modulation by VZV will be important. In all, these results identify a novel immunomodulatory mechanism employed by VZV, which supports clinical observations. This finding allows future work to focus on understanding the mechanisms of VZV-mediated CD1d downregulation and the functional consequences for iNKT cells.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Author contributions. A. A. and B. S. conceived the study. R. T., A. A., and B. S. designed the study. T. O. and R. T. undertook key experiments, analyzed data, and provided interpretation. R. T. drafted the manuscript. All authors contributed to the final version of the manuscript and provided approval of the submitted version.

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