Varicella Zoster Virus Downregulates Programmed Death Ligand 1 and Major Histocompatibility Complex 1 in Human Brain Vascular Adventitial Fibroblasts, Perineurial Cells and Lung Fibroblasts

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**ABSTRACT**

Varicella zoster virus (VZV) vasculopathy produces stroke, giant cell arteritis and granulomatous aortitis and develops after virus reactivates from ganglia and spreads transaxonally to arterial adventitia, resulting in persistent inflammation and pathological vascular remodeling. The mechanism(s) by which inflammatory cells persist in VZV-infected arteries is unknown; however, virus-induced dysregulation of programmed death ligand 1 (PD-L1) may play a role. Specifically, PD-L1 is expressed on virtually all nucleated cells and suppresses the immune system by interacting with the programmed cell death protein receptor 1 found exclusively on immune cells; thus, downregulation of PD-L1 may promote inflammation as seen in some autoimmune diseases. Both flow cytometry and immunofluorescence analyses to test whether VZV infection of adventitial cells downregulates PD-L1 showed decreased PD-L1 expression in VZV-infected compared to mock-infected human brain vascular adventitial fibroblasts (HBVAFs), perineurial cells (HPNCs) and fetal lung fibroblasts (HFLs) at 72 hours post-infection. Quantitative RT-PCR analyses showed no change in PD-L1 transcript levels between mock- and VZV-infected cells, indicating a post-transcriptional mechanism for VZV-mediated downregulation of PD-L1. Flow cytometry analyses showed decreased major histocompatibility complex 1 (MHC-1) expression in VZV-infected cells and adjacent uninfected cells compared to mock-infected cells. These data suggest that reduced PD-L1 expression in VZV-infected adventitial cells may potentially contribute to persistent vascular inflammation observed in virus-infected arteries from patients with VZV vasculopathy, while downregulation of MHC-1 prevents viral clearance.
IMPORTANCE

Here, we provide the first demonstration that VZV downregulates PD-L1 expression in infected HBVAFs, HPNCs and HFLs, which together with the noted VZV-mediated downregulation of MHC-1, might foster persistent inflammation in vessels leading to pathological vascular remodeling during VZV vasculopathy, as well as persistent inflammation in infected lungs to promote subsequent infection of T cells and hematogenous virus spread. Identification of a potential mechanism by which persistent inflammation in the absence of effective viral clearance occurs in VZV vasculopathy and VZV infection of the lung is a step toward targeted therapy of VZV-induced disease.
INTRODUCTION

Varicella zoster virus (VZV) is a common, neurotropic DNA alphaherpesvirus that causes varicella (chickenpox) upon primary infection, after which virus becomes latent in ganglionic neurons along the entire neuraxis (1-6). With a decline in VZV-specific cell-mediated immunity, VZV reactivates in elderly or immunocompromised individuals, most commonly causing zoster (shingles). VZV can also reactivate and spread transaxionally to cerebral arteries to produce stroke with or without rash (VZV vasculopathy). Recent studies show that aside from stroke, VZV vasculopathy presents as giant cell arteritis which is the most common cause of systemic vasculitis in the elderly (7), as well as granulomatous aortitis (8).

Studies of VZV-infected cerebral and temporal arteries from patients with VZV vasculopathy show viral antigen predominantly in the outermost adventitia in early infection, supporting deposition of virus from nerve fibers that terminate within this layer followed by transmural spread (7, 9). Persistent inflammation with a predominance of T cells and macrophages can be seen even up to 10 months after disease onset and is associated with pathological vascular remodeling (10). The mechanism(s) by which inflammatory cells persist to cause vascular damage in VZV-infected arteries is unknown.

Programmed cell death ligand 1 (PD-L1), a 40-kDa type 1 transmembrane protein in the B7 immunoglobulin family that can be expressed on virtually all nucleated cells (11),
suppresses the immune system through interaction with its receptor, programmed cell
death protein 1 (PD-1) expressed on activated T cells, B cells, and macrophages. The
interaction between PD-L1 and PD-1 leads to the exhaustion of immune cells and
promotion of apoptosis in these cells. In cancer, induction of PD-L1 is a common
occurrence that can prevent immune clearance of malignant cells. Similarly, viruses
such as human immunodeficiency virus (HIV) can induce PD-L1 in immune cells to
avoid immune clearance (12). In contrast, several autoimmune diseases, such as
systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), frequently lead to
downregulation of PD-L1 and PD-1 expression, producing persistent inflammation (13).

To test whether downregulation of PD-L1 on target cells might play a role in VZV
vasculopathies, we examined VZV-induced alterations of this protein in: (i) primary
human brain vascular adventitial fibroblasts (HBVAFs), which are among the initial cells
infected following virus deposition in the artery and are key regulators of vascular tone
and function; (ii) primary human perineurial cells (HPNCs), which surround the nerve
fiber and form a barrier between the nerve and adventitial fibroblasts; and, (iii) human
fetal lung fibroblasts (HFLs), which are infected during respiratory spread of VZV upon
primary infection.
MATERIALS AND METHODS

Cell culture. HBVAFs (Sciencell, Carlsbad, CA), HPNCs (Sciencell) and HFLs (ATCC, Manassas, VA) were seeded at a density of 2,000 cells/cm² in basal fibroblast medium supplemented with 2% fetal bovine serum (FBS), 1% fibroblast growth serum and 1% 100x penicillin-streptomycin (Sciencell). After 24 h, the medium was changed to basal fibroblast medium supplemented with 0.1% FBS and 1% 100x penicillin-streptomycin that was replenished every 48-72 h for 6-7 days to establish quiescence. At day 7, quiescent HBVAFs, HPNCs and HFLs were co-cultivated with the respective VZV-infected (25-30 pfu/ml) or uninfected (mock-infected) cells; as a positive control for MHC-1 and PD-L1 induction, uninfected quiescent HBVAFs and HFLs were also treated with tumor necrosis factor-α (TNFα, 100 ng/ml; Millipore, Billerica, MA) for 24 h, and HPNCs were treated with interferon-gamma (IFNγ, 100 ng/ml; Cell Signaling, Danvers, MA) for 24 h. Cells were harvested using sodium citrate (14) to optimize detection of cell surface proteins by flow cytometry analysis at 24 h post-infection (hpi) for TNFα- and IFNγ-treated cells and at 24 and 72 hpi for mock- and VZV-infected cells.

Flow cytometry (FACS). Mock- and VZV-infected cells at 24 and 72 hpi were washed with FACS buffer (phosphate-buffered saline containing 1% FBS) and stained with mouse anti-human PD-L1 antibody (APC; eBioscience, San Diego, CA), mouse anti-human MHC-1 antibody (PE-Cy7; eBioscience) and mouse anti-VZV-gE (Millipore) antibody conjugated to R-phycoerythrin using the SiteClick Antibody labeling kit (ThermoFisher, Waltham, MA) for 30 min at 4°C, washed with FACS buffer and fixed.
with 1% paraformaldehyde. Fluorescence-minus-one (FMO) and isotype controls were used in all stainings. Cells were analyzed using a Canto-II or LSR-II flow cytometer (BD Immunocytometry Systems, San Jose, CA); > 15,000 events were collected for all samples. Electronic compensation was performed with antibody capture beads (BD Biosciences, San Jose, CA), with subsequent data analyzed using Diva software (BD Biosciences) and FlowJo Software (Tree Star, Ashland, OR).

Quantitative PCR analysis. Total RNA was extracted using Trizol reagent (Thermo Fisher, Waltham, MA) at 72 hpi from mock- and VZV-infected cells; RNA was extracted from IFNγ/TNFα-treated cells after 24 h of treatment. Residual DNA was enzyme-degraded using the Turbo-DNA free kit (Thermo-Fisher, Grand Island, NY). First-strand cDNA synthesis was carried out using the Transcriptor first-strand cDNA synthesis kit (Roche, San Francisco, CA). Primers specific for PD-L1 (15) and RPL13a (16) were used for amplification and results were analyzed using the ΔΔCT method. Gene expression is represented as fold-change in virus-infected versus mock-infected cells or cytokine-treated versus mock-infected cells.

Immunofluorescence. HFLs, HBVAFs, and HPNCs were propagated on coverslips, grown to quiescence, mock-infected, VZV-infected or treated with cytokines as described above, and fixed using 4% paraformaldehyde. Cells were stained with mouse anti-human VZV-gE (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-human PD-L1 (Abcam, Cambridge, MA). Secondary antibodies consisted of Alexa Fluor 594 donkey anti-mouse IgG or Alexa Fluor 488 donkey anti-rabbit IgG (Life-
Technologies, Grand Island, NY). Coverslips were mounted with Vectashield containing DAPI (Vector Labs, Burlingame, CA) and visualized by light microscopy.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism (GraphPad, San Diego, CA). Statistical significance was determined using the Student's paired t-test.

**RESULTS**

**VZV-mediated downregulation of PD-L1 and MHC-1 expression in primary HFLs, HBVAFs, and HPNCs.** In 6 independent experiments, PD-L1 mean fluorescence intensity (MFI) at 24 hpi did not differ significantly among VZV-infected HFLs, uninfected bystander HFLs and mock-infected HFLs (923, 870 and 907, respectively) (Fig. 1A and B) or among VZV-infected HBVAFs, uninfected bystander HBVAFs and mock-infected HBVAFs (591, 564 and 555, respectively) (Fig. 2A and B), but was significantly increased in VZV-infected HPNCs compared to mock-infected HPNCs (1187 and 926, respectively; P = 0.04) (Fig. 3A and B). At 72 hpi, PD-L1 MFI in VZV-infected HFLs was significantly decreased (665) as compared to uninfected bystander (792, P = 0.03) or mock-infected cells (835; P = 0.02). Similarly, PD-L1 MFI was significantly decreased in VZV-infected HBVAFs (296) compared to uninfected bystander (545, P = 0.009) or mock-infected cells (532, P = 0.009), as well as in VZV-infected HPNCs (619) compared to uninfected bystander (942, P = 0.004) or mock-infected cells (913, P = 0.002).
At 24 hpi, PD-L1 MFI in positive control TNFα−treated HFLs, positive control TNFα-tREATED HBVAFs and positive control IFNγ-treated HPNCs was significantly increased (2165, P < 0.01; 1,268, P < 0.001; and 2968, P < 0.001, respectively) compared to all cells analyzed at both 24 and 72 hpi. Flow cytometry analyses of PD-L1 expression at 72 hpi in mock- (blue) or VZV+ (red) cells as well as cytokine-treated cells (orange) was displayed using histograms to show total PD-L1 downregulation (Fig. 1C, 2C and 3C).

In the same experiments described above, there was no significant change in MHC-1 MFI at 24 hpi among VZV-infected HFLs, uninfected bystander HFLs and mock-infected HFLs (5575, 5344 and 5707, respectively) (Fig. 1D and E) or among VZV-infected HPNCs, uninfected bystander HPNCs and mock-infected HPNCs (7772, 8291 and 8450, respectively) (Fig. 3D and E), whereas MHC-1 MFI was significantly decreased in both VZV-infected HBVAFs (5532) and uninfected bystander HBVAFs (5166) compared to mock-infected HBVAFs (7492; P = 0.04 and 0.02, respectively) (Fig. 2D and E). At 72 hpi, MHC-1 MFI was significantly reduced in both VZV-infected HFLs (4139) and uninfected bystander cells (4461) as compared to mock-infected cells (7256; P = 0.0001 and 0.0007, respectively), and in both VZV-infected HBVAFs (3553) and uninfected HBVAFs (5068) as compared to mock-infected HBVAFs (7492; P = 0.003 and 0.008, respectively), as well as in both VZV-infected HPNCs (4459) and uninfected bystander cells (7028) as compared to mock-infected cells (8774; P = 0.002 and 0.03, respectively). In addition, MHC-1 MFI was significantly lower in both VZV-infected
HBVAFs and VZV-infected HPNCs as compared to their respective uninfected bystander cells (P = 0.02 and P = 0.008, respectively).

At 24 hpi, MHC-1 MFI was significantly increased in positive control HFLs, positive control TNFα–treated HBVAs, and positive control IFNγ–treated HPNCs (36,659, P < 0.01; 24,303, P < 0.001; and 39,039, P < 0.001, respectively) compared to all cells analyzed at both 24 and 72 hpi. Flow cytometry analyses of MHC-1 expression at 72 hpi in mock- (blue) or VZV+ (red) cells as well as cytokine-treated cells (orange) was displayed using histograms to show total MHC-1 downregulation (Fig. 1F, 2F and 3F).

VZV-mediated regulation of PD-L1 gene expression in HFLs, HBVAFs, and HPNCs. Compared to mock-infected cells, PD-L1 gene expression in VZV-infected HFLs decreased 0.84 ± .28-fold, while the control TNFα-treated HFLs showed a 5.96 ± .98-fold increase in such expression (P < 0.01) (Fig. 4A). VZV-infected HBVAFs and TNFα-treated HBVAFs showed a 1.06 ± .21-fold and a 7.56 ± 4.12-fold increase, respectively, in PD-L1 gene expression compared to mock-infected cells (P < 0.01) (Fig. 4B). In VZV-infected HPNCs, PD-L1 gene expression was decreased 0.87 ± 0.63-fold but was increased 40.59 ± 11.64-fold in IFNγ-treated HPNCs compared to mock-infected cells (P < 0.01) (Fig. 4C). These results indicate that the VZV-mediated inhibition of PD-L1 expression occurs at the post-transcriptional level.

Immunofluorescence analysis of PD-L1 and VZV gE expression in mock- and VZV-infected HFLs, HBVAFs and HPNCs. At 72 hpi, mock- and VZV-infected HFLs,
HBVAFs and HPNCs were analyzed by dual immunofluorescence using a rabbit anti-PD-L1 antibody and a mouse anti-VZV gE antibody. Mock-infected HFLs, HBVAFs and HPNCs expressed PD-L1 (green) but not VZV gE (red) (Fig. 5A, rows 1, 3 and 5, respectively), whereas VZV-infected HFLs, HBVAFs and HPNCs expressed VZV gE with minimal/no expression of PD-L1 (Fig. 5A, rows 2, 4 and 6, respectively). Positive controls for PD-L1 expression (green) were provided by HFLs and HBVAFs treated with TNFα and HPNCs treated with IFNγ for 24 hours before analysis (Fig. 5B, column 1). Negative controls were provided by omission of primary antibody (Fig. 5B, column 2).

DISCUSSION

Herein, we show that VZV infection of two vascular adventitial cell types, human brain vascular adventitial fibroblasts (HBVAFs) and perineurial cells (HPNCs), as well as human fetal lung fibroblasts (HFLs), downregulates expression of PD-L1 and MHC-1 within 72 hpi. Several remarkable features emerged from this study.

First, downregulation of PD-L1 in VZV-infected cells is novel in light of prior reports that virus infection upregulates PD-L1 in both immune and non-immune cells. Specifically, PD-L1 is increased in HIV-infected CD4+ T cells (12) and monocytes (17), Epstein Barr virus (EBV)-infected Hodgkin lymphoma cells, EBV-transformed lymphoblastoid cell lines (18) and EBV-infected nasopharyngeal carcinoma cell lines (19). Influenza and respiratory syncytial virus induce PD-L1 expression in human lung and pulmonary...
macrophages in an *ex vivo* model (20). Finally, PD-L1 is induced in adenovirus-infected hepatocytes and hepatitis B virus-infected hepatic carcinoma cells (21). In those instances, virus-induced upregulation of PD-L1 led to increased binding to the PD-1 receptor on immune cells, ultimately resulting in the apoptosis of immune cells and virus persistence.

No prior investigations into the role of VZV-mediated regulation of PD-L1 expression have been reported. However, studies involving herpes simplex virus type 1 (HSV-1), another member of the Alphaherpesvirinae subfamily, report conflicting results with regards to PD-L1 expression and viral spread in various cell types. For example, during footpad HSV-1 infection in mice, monoclonal antibody blockade of PD-L1 enhances primary and secondary CD8 T cell immune responses (22). During HSV-1 infection of murine corneas, one study demonstrated that monoclonal antibody blockade of PD-L1 reduced HSV-1-specific CD8 T cells, as well as reduced numbers of CD80-expressing dendritic cells and PD-L1+ dendritic cells, in conjunction with increased viral load (23); in another study, monoclonal antibody blockade of PD-L1 resulted in decreased apoptosis and increased proliferation and IFNγ secretion by HSV-1-specific CD4 T cells, which exasperated herpetic stromal keratitis (24).

Herein, we found that PD-L1 was downregulated in the VZV-infected vascular and lung cells, raising the possibility that apoptosis is not induced in infiltrating immune cells expressing PD-1. In addition, VZV-mediated downregulation of MHC-1 occurred prior to PD-L1 downregulation, potentially preventing presentation of viral antigens to these
infiltrating immune cells. Taken together, the virus-induced dysregulation of PD-L1 and MHC-1 may contribute to ineffective viral clearance and persistent inflammation which has been observed in virus-infected arteries of patients with VZV vasculopathy. Examination of cerebral arteries from patients as late as 10 months after the onset of VZV vasculopathy revealed viral antigen and persistent inflammation comprised predominantly of CD4+ T cells and macrophages (10); furthermore, adventitial inflammation was associated with an overlying thickened intima supporting the notion that inflammatory cells secrete soluble factors that contribute to pathological vascular remodeling (25, 26). Interestingly, a similar downregulation of the PD-1/PD-L1 pathway is seen in diseases characterized by persistent inflammation, such as mouse models of lupus-like glomerulonephritis/arthritis, cardiomyopathy, type I diabetes and autoimmune nephritis (27-30). In multiple sclerosis, polymorphisms that reduce PD-1 activity are associated with progressive disease (31). In experimental allergic encephalitis, genetic or pharmacological blockade of PD-1 or PD-L1 enhances activation and expansion of T cells and worsens central nervous system pathology (32, 33). Finally, in PD-1 knockout mice, brain infarcts are larger and immune infiltrates and microglial activation are greater (34). These findings in various autoimmune diseases have prompted therapeutic targeting to activate the PD-1 pathway and alleviate persistent inflammation and associated symptoms. Programmed death ligand-2 (PD-L2) is the other ligand for PD-1 which inhibits immune cell function similar to PD-L1; however, PD-L2 is mainly expressed on antigen presenting cells (11) and was minimally expressed in all cell lines analyzed herein (data not shown), therefore, we did not investigate its expression levels during VZV infection.
Second, PD-L1 transcript levels were unchanged in VZV-infected cells, whereas protein was decreased, indicating that regulation occurs post-transcriptionally. The exact mechanism(s) is unclear; however, VZV may induce proteasomal degradation of PD-L1 protein, a notion supported by a previous study showing that VZV induces proteasomal degradation of MHC-1 protein via ORF66-dependent phosphorylation that leads to retention of MHC-1 in the Golgi apparatus and subsequent degradation (35). VZV-mediated downregulation of other host proteins, such as glial acidic fibrillary protein (GFAP) in astrocytes (36), have been reported and appear to be targeted specifically by viral infection not complete shutdown of host protein synthesis. Initial reports of VZV-mediated downregulation of MHC-1 also showed that transferrin receptor expression was unchanged, indicating that VZV does not downregulate all host proteins during infection (37). Similarly, we did not observe any changes in transferrin receptor expression levels at 72 hpi in all cell lines analyzed (data not shown), further supporting VZV-specific MHC-1 and PD-L1 downregulation.

Finally, while downregulation of MHC-1 in VZV-infected cells is consistent with prior studies (35), concurrent downregulation of MHC-1 in uninfected bystander cells at 72 hpi has not been reported. This finding suggests that VZV-infected cells secrete a soluble factor, such as a cytokine, to effect downregulation of MHC-1 in adjacent, uninfected bystander cells. Indeed, previous studies have shown that TGFβ may suppress MHC expression since TGFβ-knockout mice have elevated MHC-1 and MHC-2 (38), while IL-10 downregulates MHC-2 expression in monocytes to prevent antigen
presentation (39). Overall, downregulation of MHC-1 would prevent presentation of VZV antigen to CD8 T cells that would lead to clearance of infected cells.

Together, our studies provide a potential mechanism by which persistent inflammation in the absence of effective viral clearance occurs in VZV vasculopathy and VZV infection of the lung. Specifically, VZV-mediated downregulation of PD-L1 in vascular cells may contribute to the persistence of immune cells in VZV vasculopathy, which secrete soluble factors that produce loss of smooth muscle cells, a thickened intima and stroke (9, 10). Similarly, in VZV-infected lung during primary infection, T cells have yet to encounter viral antigen, and the initial downregulation of MHC-1 may prevent clonal expansion of T cells specific for VZV. As infection persists in the lung, PD-L1 downregulation would prevent apoptosis induction in T cells via the PD-1 pathway, and VZV-infected lung cells would be primed to transmit virus to T cells to fulfill their role as the hematogenous carriers of virus to skin during varicella. Further characterization of potential differences in VZV modulation of PD-L1 expression in T cells and neurons is warranted since VZV has a tropism for T cells and establishes latency in neurons.
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REFERENCES


FIGURE LEGENDS

FIG 1  VZV-mediated downregulation of PD-L1 and MHC-1 expression in primary human fetal lung fibroblasts (HFLs). Mock- or VZV-infected HFLs at 24 and 72 hpi, as well as positive control TNFα-treated HFLs at 24 h post-treatment, were harvested and analyzed by three-color flow cytometry using anti-VZV gE and anti-PD-L1 antibodies (A-C) or anti-VZV gE and anti-MHC-1 antibodies (D-F); cells were gated using isotype and fluorescence-minus-one (FMO) controls. Flow cytometry plots (A) and corresponding bar graphs (B) after immunostaining with VZV gE and PD-L1 antibodies showed a significant reduction in PD-L1 mean fluorescence intensity (MFI) in VZV-infected (VZV+ 72h) HFLs at 72 hpi compared to adjacent uninfected cells (VZV- 72h, P = 0.03) or to mock-infected cells (mock 72h, P = 0.02). Histogram flow cytometry analyses at 72 hpi (C) shows total downregulation of PD-L1 MFI expression in VZV+ (red) cells compared to mock-infected (blue) or TNFα-treated (orange) cells. Flow cytometry plots (D) and corresponding bar graphs (E) after immunostaining with VZV gE and MHC-1 antibodies showed a significant reduction in MHC-1 MFI in VZV-infected HFLs at 72 hpi (VZV+ 72h, P = 0.0001) and adjacent uninfected cells (VZV- 72h, P = 0.0007) compared to mock-infected cells. Histogram flow cytometry analyses at 72 hpi (F) shows total downregulation of MHC-1 MFI expression in VZV+ (red) cells compared to mock-infected (blue) or TNFα-treated (orange) cells. All results shown are representative of 6 independent experiments with bar graphs representing average MFI values and error bars representing standard deviations. Statistical significance was determined using
the Student’s t-test. * P < 0.01 for HFLs treated with TNFα for 24 h compared to mock-
and VZV-infected HFLs analyzed at 24 and 72 hpi.

FIG 2 VZV-mediated downregulation of PD-L1 and MHC-1 expression in primary
human brain vascular adventitial fibroblasts (HBVAFs). Mock- or VZV-infected HBVAFs
at 24 and 72 hpi, as well as positive control TNFα-treated HBVAFs at 24 h post-
treatment, were harvested and analyzed by three-color flow cytometry using anti-VZV
gE and anti-PD-L1 antibodies (A-C) or anti-VZV gE and anti-MHC-1 antibodies (D-F);
cells were gated using isotype and fluorescence-minus-one (FMO) controls. Flow
cytometry plots (A) and corresponding bar graphs (B) after immunostaining with VZV
gE and PD-L1 antibodies showed a significant reduction in PD-L1 mean fluorescence
intensity (MFI) at 72 hpi in VZV-infected (VZV+ 72h) HBVAFs compared to adjacent
uninfected cells (VZV- 72h, P = 0.009) and to mock-infected cells (mock 72h, P =
0.009). Histogram flow cytometry analyses at 72 hpi (C) shows total downregulation of
PD-L1 MFI expression in VZV+ (red) cells compared to mock-infected (blue) or TNFα-
treated (orange) cells. Flow cytometry plots (D) and corresponding bar graphs (E) after
immunostaining with VZV gE and MHC-1 antibodies showed a significant reduction in
MHC-1 MFI in both VZV-infected HBVAFS at 24 hpi (VZV+ 24h, P = 0.04) and adjacent
uninfected cells (VZV- 24h, P = 0.02) compared to mock-infected cells. Similarly, at 72
hpi, there was a significant reduction in MHC-1 MFI in both VZV-infected HBVAFS
(VZV+ 72h, P = 0.003) and adjacent uninfected cells (VZV- 72 h, P = 0.008) compared
to mock-infected cells. There was also a significant reduction in MHC-1 MFI in VZV-
infected HBVAFs compared to adjacent uninfected cells (VZV- 72h, P = 0.02).
Histogram flow cytometry analyses at 72 hpi (F) shows total downregulation of MHC-1 MFI expression in VZV+ (red) cells compared to mock-infected (blue) or TNFα-treated (orange) cells. All results shown are representative of 6 independent experiments with bar graphs representing average MFI values and error bars representing standard deviations. Statistical significance was determined using the Student’s t-test. * P < 0.001 for HBVAFs treated with TNFα for 24 h compared to mock- and VZV-infected HBVAFs analyzed at 24 and 72 hpi.

FIG 3 VZV-mediated downregulation of PD-L1 and MHC-1 expression in primary human perineural cells (HPNCs). Mock- or VZV-infected HPNCs at 24 and 72 hpi, as well as positive control IFNγ-treated HPNCs at 24 h post-treatment, were harvested and analyzed by three-color flow cytometry using anti-VZV gE and anti-PD-L1 antibodies (A-C) or anti-VZV gE and anti-MHC-1 antibodies (D-F); cells were gated using isotype and fluorescence-minus-one (FMO) controls. Flow cytometry plots (A) and corresponding bar graphs (B) after immunostaining with VZV gE and PD-L1 antibodies showed a significant induction of PD-L1 mean fluorescence intensity (MFI) in VZV-infected HPNCs at 24 hpi (VZV+ 24h; P = 0.04) compared to mock-infected cells (mock 24h). At 72 hpi, VZV-infected (VZV+ 72h) HPNCs had a significant reduction in PD-L1 MFI compared to adjacent uninfected cells (VZV- 72h, P = 0.004) and to mock-infected cells (mock 72h, P = 0.002). Histogram flow cytometry analyses at 72 hpi (C) shows total downregulation of PD-L1 MFI expression in VZV+ (red) cells compared to mock-infected (blue) or IFNγ-treated (orange) cells. Flow cytometry plots (D) and corresponding bar graphs (E) after immunostaining with VZV gE and MHC-1 antibodies showed a
significant reduction in MHC-1 MFI in VZV-infected HPNCs at 72 hpi (VZV+ 72h, P = 0.002) and adjacent uninfected cells (VZV- 72h, P = 0.03) compared to mock-infected cells. There was also a significant reduction in MHC-1 MFI in VZV-infected HPNCs compared to adjacent uninfected cells (P = 0.008). Histogram flow cytometry analyses at 72 hpi (F) shows total downregulation of MHC-1 MFI expression in VZV+ (red) cells compared to mock-infected (blue) or IFNγ-treated (orange) cells. All results shown are representative of 6 independent experiments with bar graphs representing average MFI values and error bars representing standard deviations. Statistical significance was determined using the Student’s t-test. * P < 0.001 for HPNCs treated with IFNγ compared to mock- and VZV-infected HPNCs analyzed at 24 and 72 hpi.

FIG 4  VZV-mediated regulation of PD-L1 gene expression in mock- and VZV-infected human fetal lung fibroblasts (HFLs), brain vascular adventitial fibroblasts (HBVAFs) and perineurial cells (HPNCs). RNA was harvested at 72 hpi from mock- and VZV-infected HFLs, HBVAFs and HPNCs then analyzed by quantitative RT-PCR for PD-L1 mRNA with results normalized to RPL-13a followed by delta-delta CT analysis. As a positive control for PD-L1 gene expression, HFLs and HBVAFs were treated with TNFα and HPNCs were treated with IFNγ then harvested for RNA analysis 24 h after treatment. (A) Compared to levels in mock-infected cells, VZV-infected HFLs PD-L1 transcripts were decreased 0.84 ± .28 fold, and TNFα treated HFLs PD-L1 transcripts were increased 5.96 ± .98 fold. (B) Compared to mock-infected cells, VZV-infected HBVAFs PD-L1 transcripts were increased 1.06 ± .21 fold, and TNFα treated HBVAFs PD-L1 transcripts were increased 7.56 ± 4.12 fold. (C) Compared to mock-infected cells, VZV-
infected HPNCs PD-L1 transcripts were decreased 0.87 ± .63 fold, and IFNγ treated HPNCs PD-L1 transcripts were increased 40.59 ± 11.64 fold. Bar graphs represent the average PD-L1 fold change with error bars representing standard deviation from triplicate experiments. Statistical significance was determined using the Student’s t-test.

* P < 0.01.

**FIG 5** Immunofluorescence analysis of PD-L1 and VZV gE expression in mock- and VZV-infected human fetal lung fibroblasts (HFLs), brain vascular adventitial fibroblasts (HBVAFs) and perineurial cells (HPNCs). At 72 hpi, mock- and VZV-infected HFLs, HBVAFs and HPNCs were analyzed by dual immunofluorescence using a rabbit anti-PD-L1 antibody and a mouse anti-VZV gE antibody. (A) Mock-infected HFLs, HBVAFs and HPNCs expressed PD-L1 (green) but not VZV gE (red) (rows 1, 3, 5 and 7, respectively), whereas VZV-infected HFLs, HBVAFs and HPNCs expressed VZV gE with minimal to no expression of PD-L1 (rows 2, 4, 6 and 8, respectively).

Magnification, 600x. (B) Positive controls for PD-L1 expression (green) were provided by HFLs and HBVAFs treated with TNFα, as well as HPNCs treated with IFNγ for 24 hours before analysis (column 1). Negative controls were provided by omission of primary antibody (column 2). Blue color represents DAPI cell nuclei stain.

Magnification, 400x.
Figure 2

A

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24 hpi

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C

10^2 10^3 10^4 10^5

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E

24 hpi

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F

10^2 10^3 10^4 10^5

VZV TNFα

VZV gE

MHC-1

24 hpi

72 hpi

Mock

VZV

VZV gE

PD-L1

24 hpi

72 hpi

TNFα

Mock
Figure 3

A

B

C

D

E

F

**Figure 3**

**A**

IFNγ

Mock

VZV

PD-L1

VZV gE

24 hpi

72 hpi

Mock

PD-L1

Count

102 103 104 105

MHC-1 Count

103 104 105

24 hpi

72 hpi

Mock

PD-L1

Count

102 103 104 105

VZV IFNγ

VZV gE

MHC-1

24 hpi

72 hpi

Mock

PD-L1

Count

102 103 104 105

**Figure 3**

**D**

IFNγ

Mock

VZV

PD-L1

VZV gE

24 hpi

72 hpi

Mock

PD-L1

Count

102 103 104 105

MHC-1 Count

103 104 105

24 hpi

72 hpi

Mock

PD-L1

Count

102 103 104 105
Figure 5

A

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