

Varicella zoster virus impairs expression of the non-classical major histocompatibility complex class I-related gene protein (MR1)

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24 **Summary of article's main point**

25 In this study, we demonstrate that Varicella Zoster Virus (VZV) profoundly suppresses
26 expression of the antigen presentation molecule Major Histocompatibility Complex, Class I-
27 Related (MR1), thus identifying an additional immunomodulatory function encoded by this
28 virus.

29 **ABSTRACT**

30 The antigen presentation molecule MR1 presents ligands derived from the riboflavin (vitamin
31 B) synthesis pathway, which is not present in mammalian species or viruses, to Mucosal
32 Associated Invariant T (MAIT) cells. In this study, we demonstrate that Varicella Zoster
33 Virus (VZV) profoundly suppresses MR1 expression. We show that VZV targets the
34 intracellular reservoir of immature MR1 for degradation, whilst pre-existing, ligand bound
35 cell surface MR1 is protected from such targeting; thereby highlighting an intricate temporal
36 relationship between infection and ligand availability. We also identify VZV open reading
37 frame (ORF) 66 as functioning to suppress MR1 expression when this viral protein is
38 expressed during transient transfection, but this is not apparent during infection with a VZV
39 mutant virus lacking ORF66 expression. This indicates that VZV is likely to encode multiple
40 viral genes that target MR1. Overall, we identify an immunomodulatory function of VZV
41 whereby infection suppresses the MR1 biosynthesis pathway.

42

43 **Keywords:** varicella zoster virus, immune modulation, MR1

BACKGROUND

Innate-like T cells often express a semi-invariant T cell receptor (TCR) and are restricted by monomorphic MHC-I-like molecules, allowing for rapid responses to conserved antigens [1]. Major Histocompatibility Complex, Class I-Related (MR1) is a monomorphic β_2 microglobulin (β_2m)-associated antigen presenting molecule that is highly conserved across the mammalian spectra and transcribed across diverse cell lineages [2, 3]. MR1 binds to unstable neo-antigenic metabolites derived from riboflavin (vitamin B) synthesis [4, 5]; a conserved pathway shared by diverse bacterial and fungal pathogens and commensals that is not present within mammals or viruses. Unlike conventional major histocompatibility complex (MHC) molecules, that constitutively bind to endogenous ligands, MR1 is predominantly sequestered in the endoplasmic reticulum (ER) in the steady state that is normally barely detected on the plasma membrane [6]. During this steady state, MR1 remains partially folded in a ligand-receptive conformation, acting as an ER resident antigen sensor [6]. Binding of ligand with intracellular MR1 triggers a molecular switch, allowing for complete MR1 folding, β_2m association and transport to the cell surface [6] where it presents metabolic antigens to Mucosal Associated Invariant T cells (MAIT cells) [5].

MAIT cells are an abundant unconventional T cell population [7], and typically express a semi-invariant T cell receptor (TCR) that allows their restriction to MR1 [8, 9]. The cognate interaction between antigen loaded, surface-expressed MR1 and the MAIT TCR results in the rapid activation of MAIT cells and allows for a functionally reactive, pro-inflammatory response against a diverse range of riboflavin synthesizing pathogens [10-12].

Whilst viruses do not synthesize vitamin B metabolites, viral infections can disrupt mucosal integrity, increasing the risk of bacterial translocation, secondary infections and altered

availability of the MR1 ligand. We previously demonstrated that herpes simplex virus 1 (HSV-1) and human cytomegalovirus (HCMV) suppress MR1 cell-surface expression [13]. Furthermore, suppression of MR1 by HSV-1 inhibited MR1-dependent activation of Jurkat cells expressing the MAIT TCR [14].

Here, we provide evidence that varicella zoster virus (VZV) profoundly disrupts cell-surface MR1. We demonstrate that VZV targets intracellular reservoirs of immature MR1, whilst pre-existing ligand-bound surface MR1 is protected from VZV-mediated targeting. Furthermore, we identify VZV ORF66 as functioning to suppress surface MR1. The results identify a new immunomodulatory function of VZV and contribute to the emerging field of virus-mediated modulation of the MR1 biosynthesis pathway.

METHODS

Cells

ARPE-19 MR1-GFP retinal pigment epithelial cells were engineered to overexpress MR1 with enhanced green fluorescence protein (EGFP) fused to the MR1 C-terminus [13]. ARPE-19 MR1 cells overexpress MR1 with EGFP expressed under the same promoter via a downstream internal ribosome entry sequence (IRES) [13]. Human embryonic kidney 293 (HEK 293T) cells were used for transfection experiments. All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Lonza) supplemented with 10% Foetal Calf serum (FCS) (Sigma Aldrich) and 1% Penicillin/Streptomycin (P/S) (Gibco).

MR1 ligands and treatment

Synthetically derived MR1 ligand: Acetyl-6-formylpterin (Ac-6-FP) (Schirks Laboratories 29769-49-1) was added to media (5 μ M) to induce MR1 surface expression. During infection

experiments target cells were treated with Ac-6-FP either 24 hours prior to VZV infection, or post VZV infection at 16 or 40 hpi or left untreated. Cells were harvested at 20 or 44 hpi. During transfection experiments cells were treated with Ac-6-FP 24 hours post transfection and harvested 28 hours post transfection.

Viruses and infection

Clinical strain (VZV-S), VZV recombinant Oka strain (rOka), and VZV rOka-ORF66s (ORF66s; which is unable to express the ORF66 protein) [15] were passaged in ARPE-19 cells. VZV infected ARPE-19 cells at a cytopathic effect (CPE) of 3+ to 4+ (defined as 70 to 100% of the monolayer showing CPE) were added to uninfected target cells at a ratio of 1:1 and incubated at 37°C 5% CO₂.

Plasmid constructs and transfection

Plasmids pGK2-ORF66-HA and pGK2-ORF47-HA encode VZV ORF 66 and 47 respectively, fused to an amino terminal haemagglutinin (HA) tag under the control of the HCMV IE promoter. HEK 293T cells were transfected with 5µg of parental plasmid pGK2-HA, pGK2-ORF66-HA or pGK2-ORF47-HA using FuGene HD (Promega).

Flow cytometry

For infection experiments, VZV infected ARPE-19 cells were stained with Cell Trace Violet (CTV) (Invitrogen) as described [16]. After harvest, cells were stained with Zombie NIR fixable viability dye (BioLegend), and with antibodies: anti MR1-APC (clone 26.5, BioLegend), anti HLA-ABC-APC (clone G46-2.5, BD Biosciences), anti gE:gI (clone SG1, Meridian Life Sciences) conjugated in-house to PE, or isotype controls. Cells were fixed

using Cytofix (BD Biosciences), which does not permeabilize cells. Flow cytometry was performed using an LSR II (BD Biosciences).

In transfection experiments, cells were stained with antibodies: anti HLA-ABC-APC (clone G46-2.5, BD Biosciences) or anti MR1-Biotin (clone 26.5) followed by streptavidin-PE (eBioscience). Cells were fixed and permeabilized using Cyto Perm/Fix (BD Biosciences) and incubated with anti HA-Alexa Fluor 488 (clone 912426, R&D Systems). Isotype controls were included. Samples were acquired using an LSR Fortessa X-20 flow cytometer (BD Biosciences).

Flow cytometry data analysis

Flow cytometry data was analysed using FlowJo software version 10.0.6 (FlowJo, USA). For infection experiments, CTV and Zombie positive cells were gated to exclude inoculum and dead cells, respectively, and then gated for gE:gI expression to confirm VZV surface antigen expression in infection (Supplementary Figure 1). For transfection experiments, cells were gated for HA expression to identify VZV-ORF expressing cells. To facilitate comparison of relative fluorescence intensity, the X-axis is consistent in all figures other than Figure 5 (where endogenous surface MR1 was measured).

Immunoblotting and Endo H digestion

ARPE-19 MR1 overexpressing cells were infected with VZV-S infected ARPE-19 cells, or mock inoculated. Target cells were treated with Ac-6-FP ligand. Lysates were harvested at 20 or 44 hpi and then Endo H (NEB) digested as per the manufacturer's instructions. Proteins were resolved by SDS-PAGE. Membranes were probed with anti-MR1 (#55164, Abcam), anti-VZV IE62 (clone number IE62, Meridian) or anti-actin (#A2066, Sigma Aldrich)

followed by an HRP-conjugated secondary antibody (Santa Cruz Biotechnology). Bands were visualized by chemiluminescence using ChemiDoc MP Imager (Bio-Rad).

RESULTS

VZV infected cells downregulate cell-surface MR1

Whilst it is well-established that VZV downregulates MHC-I expression [17], the impact of this virus on the MHC class I-related molecule MR1 has not been previously examined. As endogenous MR1 surface expression is extremely low, we utilized ARPE-19 cells engineered to overexpress MR1 fused to EGFP (ARPE-19 MR1-GFP) [6, 13] to facilitate the interrogation by flow cytometry of both surface and total MR1. VZV is highly cell-associated *in vitro*, thus a well characterized cell-associated infection model was used, whereby CTV-labelled VZV-S infected ARPE-19 cells (inoculum) were co-cultured for 20 or 44 hours with uninfected ARPE-19 MR1-GFP target cells [16] in a ligand-free assay. There was negligible cell death at both timepoints post infection, with >95% cell viability, and this remained consistent between mock and VZV infected cultures.

Due to the asynchronous nature of a cell-associated infection, uniformly high expression of VZV heterodimer glycoprotein E: glycoprotein I (gE:gI) on target cell surface was only apparent at the later timepoint (Supplementary Figure 1). By contrast, gE:gI expression was variable at 20 hours post infection (hpi), likely reflecting cells at various stages of infection. Indeed, initial analysis of the total gE:gI positive population of target infected cells at 20 hpi revealed no significant difference in surface MR1 expression when compared to mock

infected ARPE-19 MR1-GFP cells (data not shown). However, gating target cells at 20 hpi into low and high gE:gI sub-populations permitted us to separately analyse the capacity of VZV to modulate MR1 in these cells.

High gE:gI expressing target cells at 20 hpi demonstrated potent downregulation of surface MR1 in comparison to mock inoculated target cells (Figure 1A). By contrast, target cells expressing low gE:gI at 20 hpi demonstrated no difference in surface MR1 compared to mock (Figure 1A). At 44 hpi, where the population of high gE:gI expressing target cells comprised the vast majority of infected target cells, MR1 surface expression remained profoundly downregulated (Figure 1A). We also examined surface expression of the related molecule, MHC-I. Significant downregulation of surface MHC-I was only observed at 44 hpi (Figure 1B), consistent with other studies [18]. These findings indicate that VZV infection inhibits surface MR1 expression.

To confirm that VZV infection was unaffected by the MR1-GFP expression construct, CTV-labelled VZV-S infected ARPE-19 cells (inoculum) were cocultured with the parental ARPE-19 cell line. No differences were observed in VZV infection of the parent ARPE-19 cells compared to those transduced to overexpress MR1, either in terms of the timing or extent of CPE, or in the extent of gE:gI staining.

Ligand pre-treatment protects surface MR1 from VZV induced downregulation

MR1 ligand availability triggers a ‘molecular switch’ allowing for rapid intracellular trafficking and surface expression of the MR1-antigen complex [6]. The impact of ligand availability on surface MR1 during VZV infection was investigated. VZV-S infected ARPE-19 cells were co-cultured for 20 or 44 hours with uninfected ARPE-19 MR1-GFP target cells. Synthetic MR1 ligand Ac-6-FP was added either 24 hours prior to VZV infection, or post-

VZV infection for the final four hours prior to staining. Surprisingly, cells pre-treated with ligand prior to infection demonstrated surface MR1 levels comparable to mock control at both 20 and 44 hpi (Figure 2A). However, high gE:gI expressing target cells treated with ligand post-VZV infection demonstrated significantly less surface MR1 at both timepoints compared to mock (Figure 2A). As expected, VZV modulation of surface MHC-I was unaffected by MR1 ligand availability (Figure 2B). These results reveal a temporal relationship between ligand availability and VZV infection whereby only pre-existing cell surface MR1 following treatment with ligand is protected from VZV targeting during infection. We have also observed that ligand pre-treatment protects surface MR1 from targeting during HSV-1 infection [13], suggesting that this may be a conserved outcome of alphaherpesvirus infection.

VZV infection downregulates total MR1

To further examine the impact of VZV on MR1 protein expression, we evaluated whether the observed ligand-dependent modulation of surface MR1 reflected relative levels of total cellular MR1, as detected by GFP fluorescence. GFP expression from ARPE-19 MR1-GFP target cells was examined at 20 and 44 hpi in the absence of ligand, as well as with ligand addition prior to and post-infection. High gE:gI expressing target cells downregulated total MR1-GFP at 20 hpi across all ligand conditions (Figure 3A). Low gE:gI expressing target cells did not display a significant change in total MR1-GFP expression levels (Figure 3A), corresponding with their observed inability to significantly downregulate surface MR1 (Figures 1A and 2A). Furthermore, the significant decrease in total MR1 (as reflected by MR1-GFP) observed by high gE:gI expressing cells at 20 hpi was also observed at 44 hpi (Figure 3B). At both timepoints there was significantly more MR1-GFP with ligand pre-

treatment of high gE:gI expressing infected cells compared to those cells lacking ligand or receiving Ac-6-FP post infection. These data demonstrate that VZV infection limits the pool of MR1 available for trafficking to the cell surface and this is partially inhibited when ligand is available prior to infection.

Mature Endo H resistant MR1 is resistant to VZV targeting

Upon ligand binding, ER-resident MR1 is presumed to undergo conformational change and glycosylation maturation as it traffics through the trans golgi network (TGN) to the cell surface [6]. To determine whether all these forms of MR1 are targeted by VZV, mock and VZV infected ARPE-19 MR1 cells (which overexpresses untagged MR1) [13] were treated with Ac-6-FP before or after infection, or left untreated. Cell lysates were harvested at 20 and 44 hpi and digested with Endo H before immunoblotting to separately identify ER-resident, Endo H sensitive forms of MR1 from their mature, Endo H-resistant counterparts that have been processed in the TGN [6]. In the absence of ligand, very little higher molecular weight Endo H-resistant MR1 was detected (Figures 4A and 4B), with the lower molecular weight, ER-resident, Endo H-susceptible form dominating. However, samples treated with ligand both prior to and after mock infection demonstrated increased Endo H-resistant MR1 reflecting the effect of ligand on MR1 maturation (Figures 4A and 4B). Infection of cells was validated by immunoblotting for VZV encoded Immediate Early IE62 protein at 20 hpi and 44 hpi, and for actin as a loading control (Figure 4A and 4B). At 20 hpi the reduction in both forms of MR1 in the VZV inoculated samples was modest, reflecting the presence of a mixture of uninfected target cells and those at early and late stages of infection. However, by 44 hpi there was almost a complete loss of all forms of MR1 in cells treated with ligand post-infection, whilst in cells pre-treated with ligand before infection, there was evidence of protection of the mature Endo H-resistant band but not the Endo-H sensitive band (Figure

4B). This data suggests an ability of VZV to selectively target immature MR1 and provides evidence in support of the data from Figure 1A, that VZV does not efficiently target pre-existing complex-glycosylated MR1.

VZV ORF66 expression partially modulates surface MR1 expression

Given the involvement of VZV encoded serine kinase ORF66 in downregulating MHC-I [17, 19], and the capacity of its HSV-1 homolog Us3 to modulate surface MR1 [13], we determined whether ORF66 downregulated MR1. In addition to ORF66, we also examined a second VZV encoded serine threonine kinase, ORF47, which does not modulate surface MHC-I [20].

HEK 293T cells were transfected with plasmids expressing HA tagged ORF66 or ORF47 (pGK2-HA66 or pGK2-HA47, respectively) [21] or the parental plasmid (pGK2-HA). Cells were transfected for 28 hours with addition of MR1 ligand (Ac-6-FP) 4 hours prior to harvest. Based on Mean Fluorescence Intensity, there was no significant difference in the relative expression of the ORF47-HA and ORF66-HA expression, indicating comparable levels of expression of each viral protein.

In comparison to parental plasmid, cells expressing ORF66 demonstrated a significant reduction of surface MR1 (Figure 5A). In contrast, cells expressing ORF47 did not downregulate surface MR1 (Figure 5A). Consistent with previous findings, a significant loss of surface MHC-I was observed in ORF66 but not ORF47 transfected cells (Figure 5B) [17]. These data indicate that ORF66 impairs both surface MR1 and MHC-I, whereas ORF47 impacts neither cellular protein.

We next examined the capacity of a ORF66 to modulate surface and total MR1 in the context of VZV infection using an ORF66 mutant virus (rOka 66s), which is unable to express the

ORF66 protein [15]. In ARPE-19 MR1-GFP target cells, both rOka and rOka 66s robustly downregulated surface MR1 in the absence and post treatment (40 hpi) of ligand (Figure 6A). As demonstrated with VZV-wild type strain S infection (Figure 2A), surface MR1 was protected from downregulation in cells pre-treated with ligand (24 h pre-infection) during either rOka or rOka 66s infection (Figure 6A). Again, total MR1 was significantly downregulated by both viruses across all ligand treatment conditions (Figure 6B). Both parent and mutant viruses downregulated surface MHC-I across all ligand conditions (Figure 6C); a finding consistent with previous studies of MHC-I modulation [19]. Whilst our data demonstrates ORF66 as a mediator of surface MR1 downregulation, the inability to observe a rescue of MR1 expression during infection with the ORF66 mutant virus suggests that VZV likely encodes additional ORFs to achieve MR1 suppression in the context of infection.

DISCUSSION

This study identifies a novel immunomodulatory function whereby VZV suppresses the MR1 biosynthesis pathway. We demonstrate targeting of intracellular reservoirs of immature MR1 during VZV infection. The reduction in the intracellular pool of ligand receptive MR1 severely limits the ability of VZV infected cells to capture and present MR1 ligand, with a consequence being a significant reduction of ligand bound cell-surface MR1. In contrast, pre-existing ligand bound surface MR1 was resistant to targeting by VZV, highlighting a temporally sensitive relationship between ligand availability and the timing of infection. Furthermore, we identified the VZV encoded serine/threonine kinase, ORF66, as a contributor of MR1 downregulation in a transient gene expression setting, but also that it may not be the only contributor in the context of infection.

278 A common theme during primary VZV infection (varicella) is the high virus load at
279 respiratory mucosal and skin sites. These sites are also enriched with resident MAIT cells
280 which respond to potential breaches in barrier integrity by detecting aberrant levels of
281 metabolites from riboflavin synthesising commensals [9, 22, 23]. During varicella, the
282 production of vesicular lesions disrupts the skin and mucosal barriers, providing an
283 opportunity for commensals to infect the host. The riboflavin pathway is expressed in a wide
284 range bacteria including *Staphylococcus aureus*, which is a common cause of secondary
285 bacterial infections during varicella, ranging from the more frequently observed skin and soft
286 tissue infections, to rare but serious visceral infections, sepsis and toxic shock [24-26].
287 Consequently, during primary VZV infection, disruption to the mucosal barrier is likely to
288 result in bacteria co-infecting or co-locating at sites of varicella lesions, providing sources of
289 MR1 ligands. Thus, VZV modulation of MR1 antigen presentation may impact MR1-
290 dependent presentation of intracellular or extracellular derived ligands to MAIT cells. Firstly,
291 given that MR1-TCR mediated activation of MAIT cells precedes its response to cytokines
292 [27], MAIT cell activation could be delayed. In addition, it may avert the establishment of the
293 immunological synapse between antigen-bound surface MR1 and the MAIT TCR that directs
294 the targeted cytolytic killing [10] of infected cells. Finally, it may prevent the synergistic
295 effect of TCR-mediated and innate signalling that is required for sustained activation and
296 proliferation of MAIT cells [28-30]. These combined impacts on MAIT cell activation and
297 effector responses could thus provide critical time for optimal viral replication and
298 transmission to new host cells. However, whether the modulation of MR1 by VZV provides a
299 benefit to this virus requires further examination, and functional studies of MAIT cell
300 responsiveness using models of VZV and bacterial co-infections, will be important to define
301 the extent of functional consequences of MR1 modulation by VZV.

In addition to MR1-dependent stimulation, MAIT cells can be both inhibited and activated in an MR1-independent manner by cytokines [31, 32]. In this respect, the involvement of MAIT cells in various viral infections such as influenza virus, Hepatitis B virus (HBV), Hepatitis C virus and HIV has become apparent, with studies reporting altered circulating MAIT cell frequencies and functions [33-35]. In contrast, evidence exploring the role of MR1-dependent antigen presentation during viral infection remains limited. We previously reported that epithelial cells pre-treated with MR1 ligand demonstrated a significant upregulation of surface MR1 in the context of HSV-1 infection [13]. There is also evidence of *in vitro* MR1-TCR mediated MAIT cell CD107a expression and a corresponding enhancement of cytotoxic killing against HBV antigen expressing hepatocytes [36]. In addition, SARS-CoV-2 infected macrophages trigger MR1 dependent MAIT cell Granzyme B production and degranulation *in vitro* [37]. Finally, MR1 trafficking has been shown to be dependent on NF- κ B signalling [31], a pathway that is critical to establishing the anti-viral response and is actively modulated by herpesviruses such as VZV [38]. Thus, it is feasible that viral infections impact MR1 antigen expression and contribute to the MAIT cell response, rendering it a target for viral modulation.

Despite VZV targeting immature but not pre-existing mature ligand-bound surface MR1, consistent with the related alphaherpesvirus HSV-1, HCMV is able to target MR1 in cells treated with ligand both before and after infection [13]. Indeed, the different approaches employed by these viruses to modulate MHC-I leaves open the possibility that they also encode multiple disparate mechanisms to control MR1. HCMV expresses four gene products that each target various steps within the MHC-I antigen presentation pathway [39]. During VZV, but not HSV-1 infection, MHC-I molecules accumulate in the Golgi [17]. On the other hand, whilst HSV-1 ICP47 blocks peptide transport into the ER lumen [40], a functional homolog of this immunomodulatory gene is lacking in the VZV genome. Transient gene

327 expression revealed that ORF66 (Figure 5) impacts MR1 in the absence of other viral gene
328 products, whilst the ORF66s mutant failed to rescue surface MR1 or MHC-I (Figure 6),
329 implying that other VZV genes play important roles in the modulation of these immune
330 molecules during viral infection.

331 ORF66 has orthologs in all alphaherpesviruses and is considered part of the US3 family of
332 herpesvirus protein kinases. They have common features in that their targets are basophilic
333 [41] and sometimes overlap with the basophilic cellular protein kinase PKA [42]. Whilst
334 many HSV-1 Us3 targets have been identified, only a small number have been confirmed for
335 VZV ORF66, including the cellular nuclear matrix protein Matrin 3, type 1 histone
336 deacetylases and viral protein IE62 [21, 42, 43]. Until more targets are identified, the
337 predicted consensus sequence for ORF66 remains loosely defined, and thus at present
338 narrowing the list of potential cellular targets involved in MR1 downregulation remains a
339 challenge.

340 Whilst ORF66 downregulated MR1 and MHC-I, the other VZV serine/threonine kinase
341 ORF47 did not. In contrast to the basophilic ORF66, ORF47 is known to target motifs that
342 are acidic, and the nature of this target difference likely underlies the lack of ORF47 activity
343 on MHC-I, and perhaps also on MR1.

344 In conclusion, this study extends our understanding of the viral modulation of MR1 and
345 provides the first evidence that VZV suppresses MR1 biosynthesis. This underscores the
346 remarkable diversity of mechanisms employed by herpesviruses to subvert host defences.

347

348 **Conflicts of interest**

349 The authors declare no conflicts of interest.

350

351 **Author contributions**

352 S.K.P. and C.S. performed experiments. B.S., A.A., S.K.P, C.S. designed experiments and
353 analysed data. S.K. P., C.S., A.A and B.S. wrote the manuscript. H.E.G.M., P.R.K, J.A.V,
354 R.T., M.S., B.P.M., D.C.T., and J.R. provided reagents and/or technical assistance. All
355 authors read, edited and approved the manuscript.

356

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FIGURE LEGENDS

Figure 1. VZV downregulates surface MR1 at 20 and 44 hpi

ARPE-19 MR1-GFP (A) or ARPE-19 (B) target cells were inoculated with VZV-S infected ARPE-19 cells at a ratio of 1:1, or mock inoculated in parallel. At 20 and 44 hpi cells were harvested, stained for cell-surface gE:gI, MR1 (A), MHC-I (B) or isotype control antibody (grey histogram) and analysed by flow cytometry. Expression of the relevant surface marker was compared to mock (blue) in target cells expressing low (yellow) or high (red) gE:gI levels, relative Median Fluorescence Intensity (MFI) fold change over mock was calculated for surface MR1 (n=4), and MHC-I (n=3). Error bars depict the SEM, significance compared to mock was calculated by Student's *t* test. ** $p<0.01$, **** $p<0.0001$

Figure 2. Ligand pre-treatment protects surface MR1 during VZV infection at 20 and 44 hpi

ARPE-19 MR1-GFP (A) or ARPE-19 (B) target cells were inoculated with VZV-S infected ARPE-19 cells at a ratio of 1:1, or mock inoculated in parallel. Target cells were treated with Ac-6-FP ligand (5 μ M) either 24 hours prior to VZV infection, or post VZV infection at either 16 or 40 hpi (A, B). At 20 or 44 hpi, cells were harvested and stained for cell-surface gE:gI, MR1 (A), MHC-I (B) or isotype control antibody (grey) and analysed by flow cytometry. Expression of the relevant surface marker was compared to mock (blue) in target VZV infected cells expressing low (yellow) or high (red) gE:gI levels). Relative MFI fold change over mock was calculated for surface MR1 (n=4) and MHC-I (n=3). Error bars depict the SEM, significance compared to mock was calculated by Student's *t* test. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Figure 3. VZV downregulates total MR1-GFP at 20 and 44 hpi

ARPE-19 MR1-GFP cells were inoculated with VZV-S infected ARPE-19 cells at a ratio of 1:1, or mock inoculated in parallel. Target cells were treated with Ac-6-FP ligand (5 μ M) either 24 hours prior to VZV infection, or post VZV infection at either 16 hpi (**A**) or 40 hpi (**B**) or left untreated with no ligand (NL). At 20 hpi (**A**), or 44 hpi (**B**), cells were harvested, stained for surface gE:gI and analysed by flow cytometry. Expression of MR1-GFP was compared to mock (blue) in target cells expressing low (yellow) or high (red) gE:gI levels. Relative MFI fold change over mock was calculated for total MR1 (n=4). Error bars depict the SEM, significance compared to mock and between infected samples was calculated by Student's *t* test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Figure 4. Mature Endo H resistant MR1 is retained during VZV infection

ARPE-19 MR1 overexpressing cells were infected with VZV-S infected ARPE-19 cells at a ratio of 1:1, and mock inoculated in parallel. Target cells were treated with Ac-6-FP ligand (5 μ M) either 24 hours before, 16 (**A**) or 40 (**B**) hours post VZV infection, or left untreated. At 20 (**A**) or 44 hpi (**B**), cells were harvested, lysed and Endo H (+) or mock (-) digested. Proteins were separated by gel electrophoresis and stained for MR1, actin and VZV-IE62. Endo H resistant (R) and susceptible bands (S) are denoted. Images are representative of two biological repeats.

Figure 5. VZV ORF66 transient expression downregulates surface MR1

HEK 293T cells were transfected with either ORF66 (n=7) or ORF47 (n=4) expressing plasmids (pGK2-HA-66 and pGK2-HA-47, respectively) or the parental (n=7) control

plasmid (pGK2-HA). Cells were treated with Ac-6-FP ligand (5 μ M) 24 hours post transfection. Cells were harvested at 28 hours post transfection and stained for endogenous surface MR1, MHC-I, intracellular HA or isotype control (grey) and analysed by flow cytometry. After gating for HA-expression, surface expression of MR1 (A) and MHC-I (B) was compared to the parent control (blue), in the ORF66 (red) and ORF47 (green) transfected cells. Relative MFI fold change over parent control for surface MR1 (A) and MHC-I (B) was calculated. Error bars depict the SEM, significance compared to parent control was calculated by Student's *t* test. **** $p < 0.0001$

Figure 6. rOka and rOka66s similarly downregulate surface and total MR1 at 44 hpi

ARPE-19 MR1-GFP cells (A, B) or ARPE-19 cells (C) were inoculated with VZV rOka or rOka 66s infected ARPE-19 cells at a ratio of 1:1, or mock inoculated in parallel. Target cells were treated with Ac-6-FP ligand (5 μ M) either 24 hours prior to, or post VZV infection at 40 hpi (A, B, C). At 44 hpi, cells were stained for surface gE:gI, surface MR1 (A), MHC-I (C) or isotype control (grey) and analysed by flow cytometry. Surface MR1 (A), total MR1-GFP (B) and surface MHC-I expression (C) was compared to mock (blue) in rOka (purple) and rOka 66S (green) infected target cells expressing high levels of gE:gI. MFI fold change over mock was calculated for surface MR1 (n=4), total MR1-GFP (n=4) and MHC-I (n=3). Error bars depict the SEM, significance against mock was calculated by Student's *t* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Supplementary Figure 1. Flow cytometry gating strategy to evaluate VZV infected target cells

ARPE-19 MR1-GFP cells were inoculated with Cell Trace Violet (CTV) labelled ARPE-19 cells infected with VZV-S at a ratio of 1:1, or mock inoculated in parallel. Cells were

448 harvested at 20 or 44 hpi and stained for viability (Zombie NIR), surface gE:gI and surface
449 MR1. Cells were initially gated to only include Zombie⁻ CTV⁻ negative cells. Infected target
450 cells were then separated into either Low or High gE:gI expressing populations based on
451 expression relative to isotype controls (grey) and the consistently high gE:gI expression
452 evident at the later timepoint.

453

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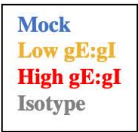
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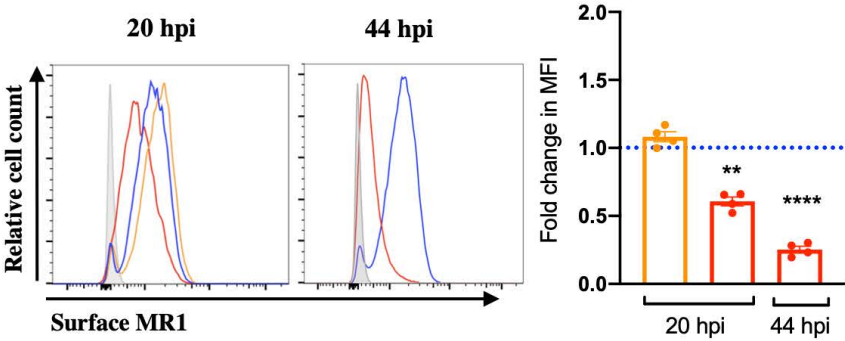
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Figure 1



A. Surface MR1



B. MHC-I

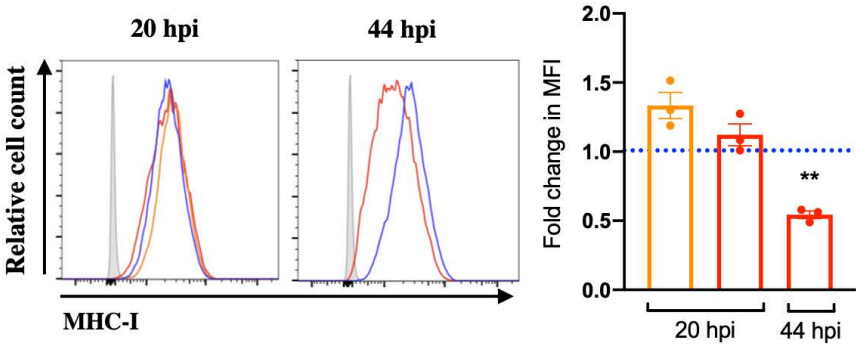
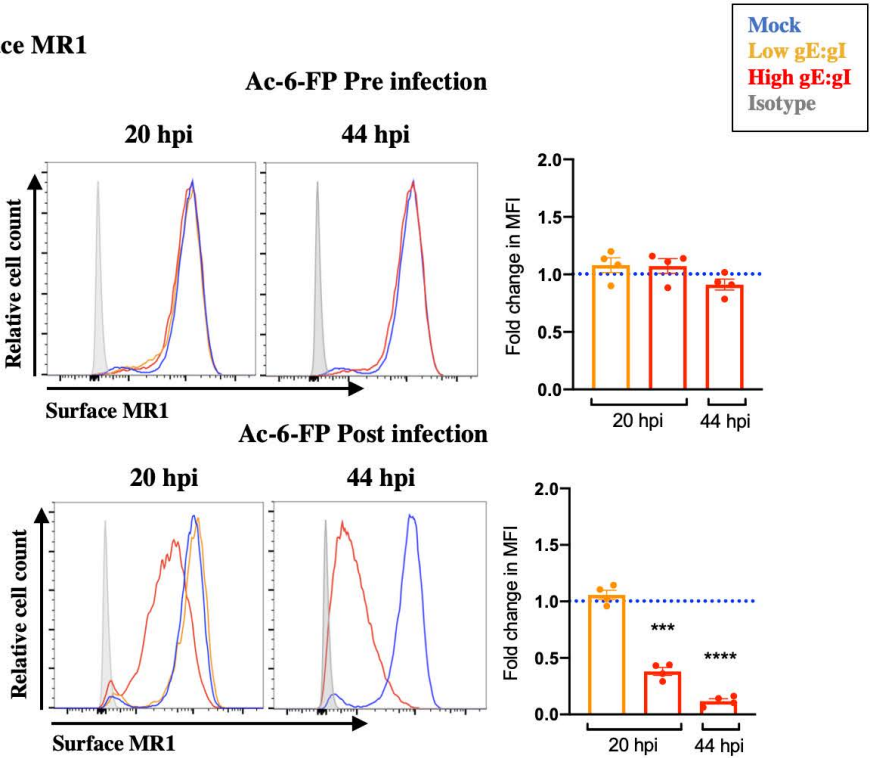


Figure 2

A. Surface MR1



B. MHC I

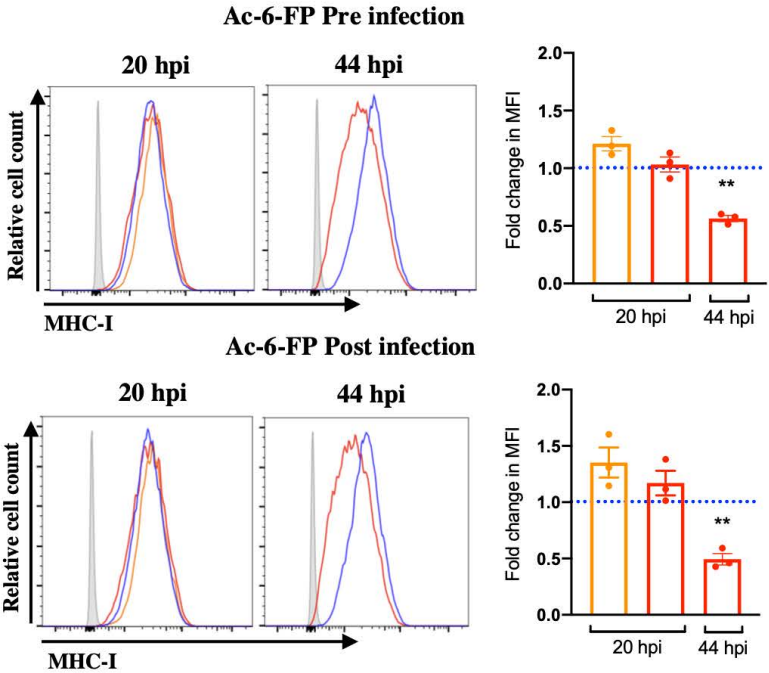
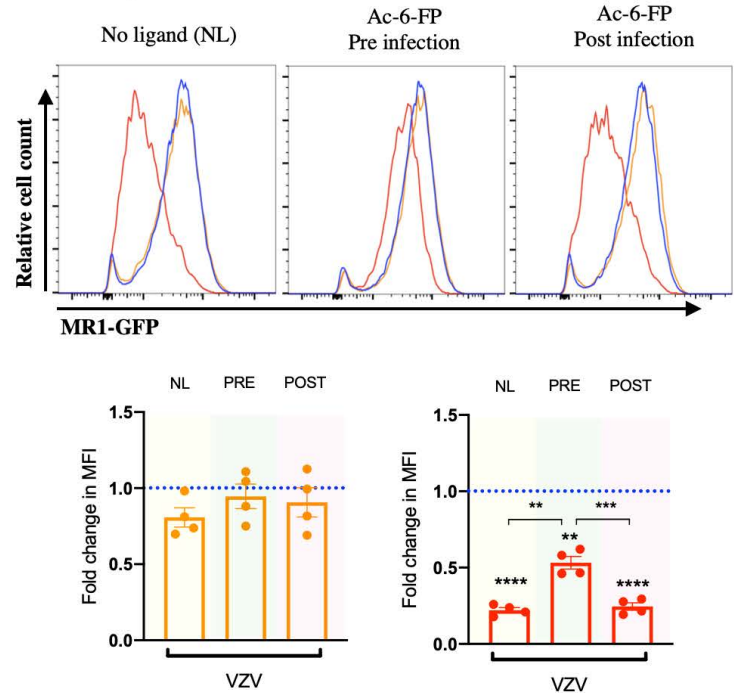


Figure 3

Mock
Low gE:gI
High gE:gI

A. Total MR1 at 20 hpi



B. Total MR1 at 44 hpi

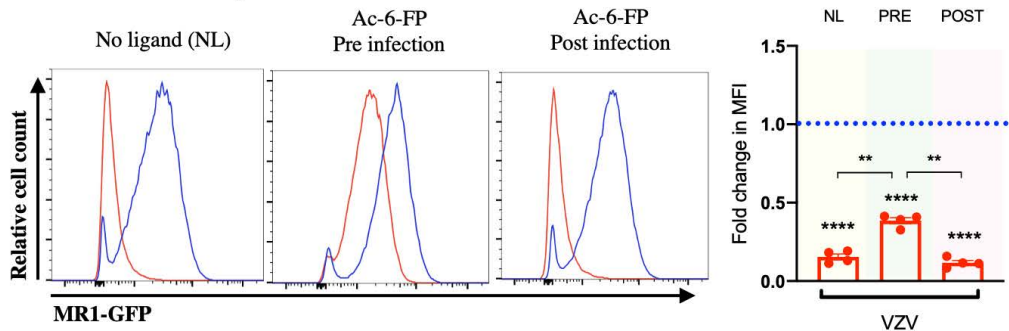
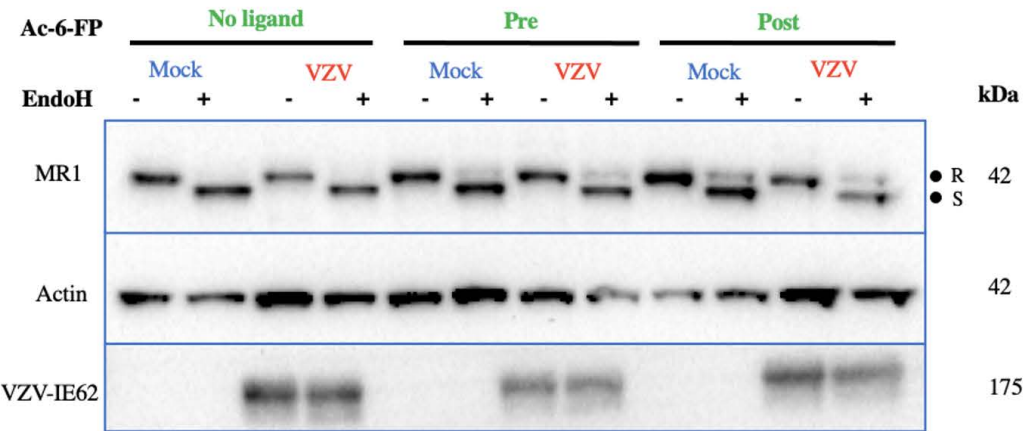


Figure 4

A. Endo H digest at 20 hpi



B. Endo H digest at 44 hpi

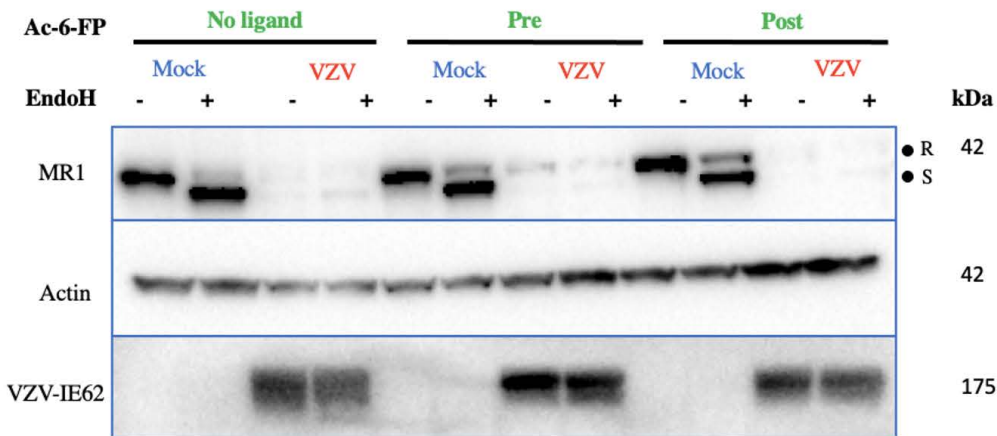


Figure 5

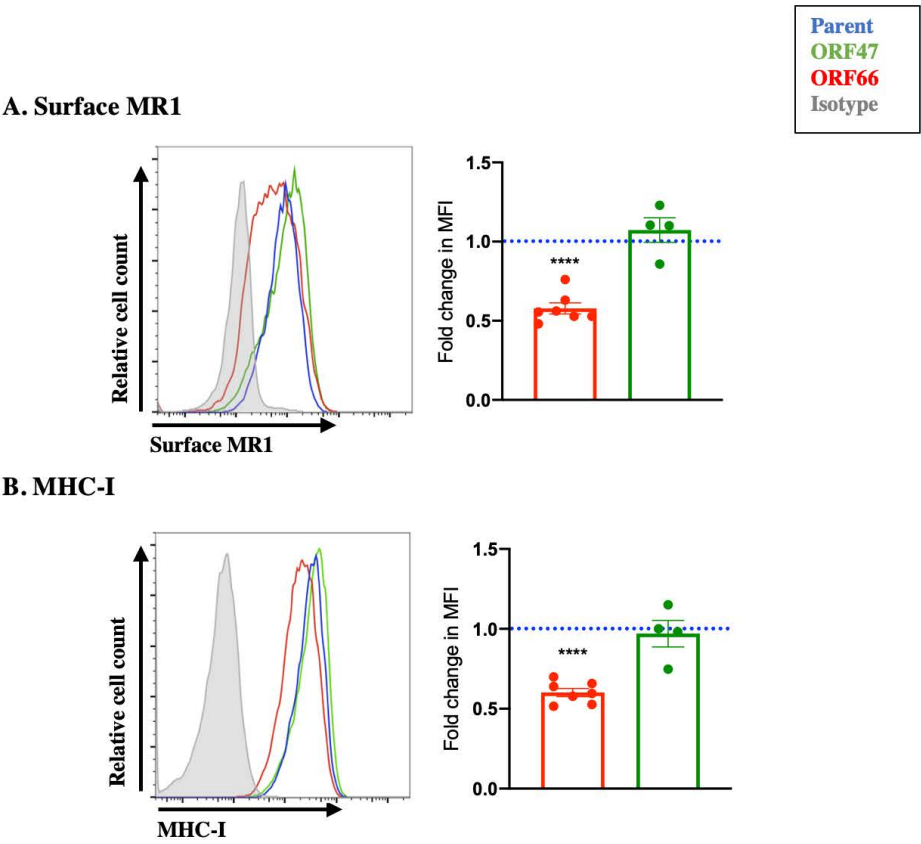
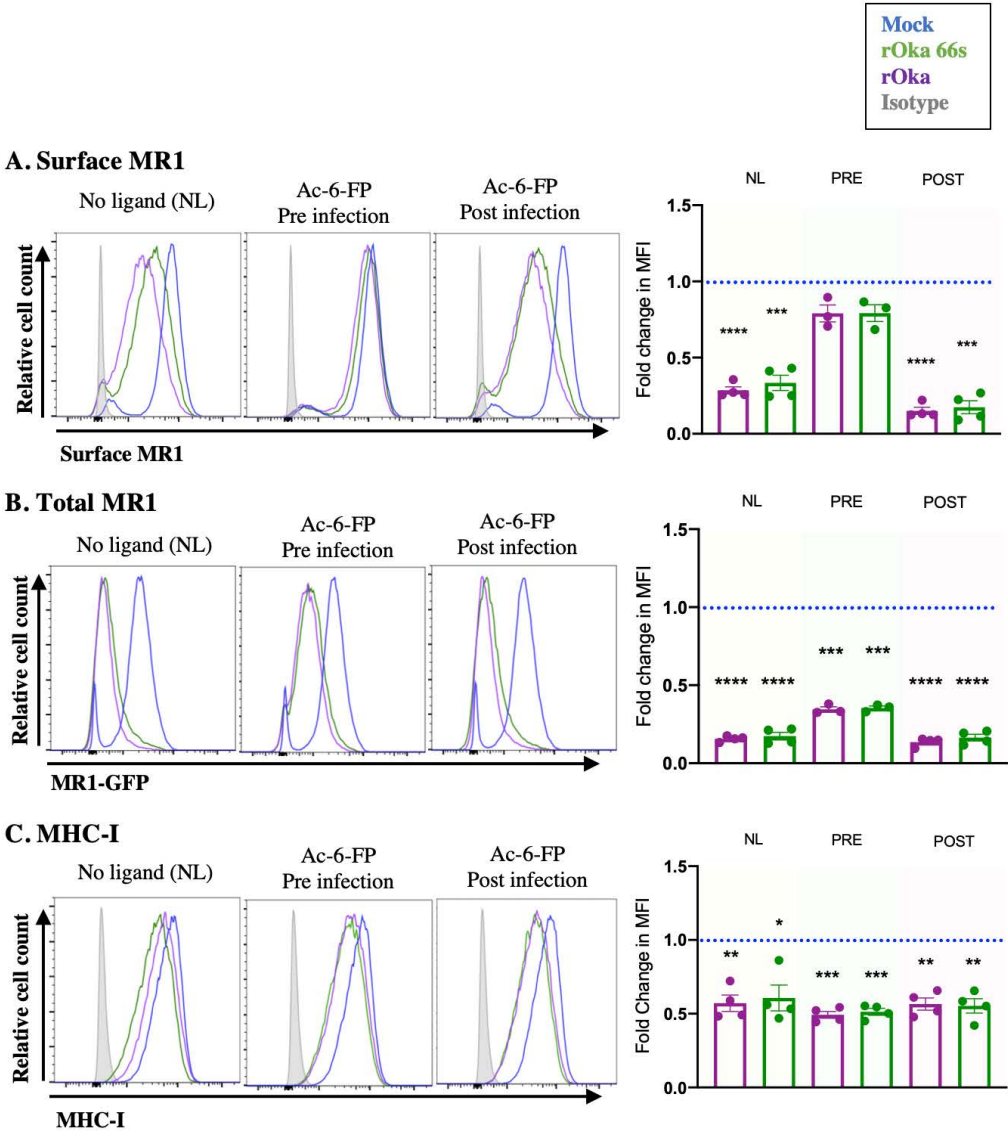


Figure 6



Supplementary Figure 1

