Profound suppression of the host MR1-MAIT cell axis by Varicella Zoster Virus

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Statement of originality

The study presented in this thesis was completed under the supervision of Professor Barry Slobedman in the Faculty of Medicine and Health at The University of Sydney.

This is to certify that to the best of my knowledge; the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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Authorship attribution statement

This thesis contains three primary research published works of which I am the first author.

Chapter 2:

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The convention for author placement is the listing of authors in order of contribution to the paper, with the senior author listed in last position.

Author contributions. S.K.P designed and conceptualised the experiments with the assistance of C.S. S.K.P performed all experiments and analysed data with the assistance of C.S. S.K.P prepared the first draft of the manuscript. Input and supervision was provided by A.A and B.S.

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In addition to the statements above, permission to include the published material has been granted by the corresponding authors.

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Shivam K Purohit Date

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

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Professor Barry Slobedman Date

Additional publications arising during candidature

Samer, C., Traves, R., **Purohit, S. K.**, Abendroth, A., McWilliam, H. E., & Slobedman, B. (2021). Viral impacts on MR1 antigen presentation to MAIT cells. *Critical Reviews™ in Immunology*, *41*(5).

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Presentations from this study

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"Varicella Zoster Virus profoundly impairs MR1 presentation pathway"
Oral presentation

2022 Charles Perkins Centre- Biodomain seminar series
"Varicella Zoster Virus hijacks Mucosal Associated Invariant T cells"
Oral presentation

2023 Charles Perkins Centre - Biodomain seminar series
"Profound viral impairment of the host metabolite antigen sensory and effector axis"
Oral presentation

Dedication and acknowledgements

Life is a funny thing.

I say this as I write these acknowledgments on an ancient work laptop that has replaced the laptop stolen from my car a week before my thesis submission. But then again, I never imagined I would be writing a thesis acknowledgments in the first place! Perhaps that is because I always felt that I was an average student. My mother would even go so far as to reassure me that an average performance "*if maintained at a consistent level*" was still an accomplishment nevertheless.

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I don't think I will ever shake this feeling of incredulity of being a scientist. I feel so incredibly lucky to be living this life. It makes me smile to think that even average individuals like myself can achieve great things through the incredible company they keep.

Abstract

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Mucosal Associated Invariant T (MAIT) cells are an unconventional immune population that functions to survey mucosal barrier and host entry sites. Through their T cell receptor (TCR), MAIT cells can respond to an evolutionarily conserved pathogen pattern of riboflavin synthesis presented by their cognately antigen presenting molecule: Major histocompatibility complex related gene protein (MR)-1. This allows the MR1-MAIT cell axis to confer resistance against and regulate host-microbiome dynamics. Furthermore, MAIT cells are innately sensitive to TCR-independent activation via local pro-inflammatory cues and thus can play a role in several viral infections. In this thesis we examine the interactions between MR1, MAIT cells and the causative agent of chickenpox (varicella) and shingles (zoster): Varicella zoster virus (VZV). Part of what allows VZV to be a globally prevalent pathogen is its ability to disarm host immune defence responses designed to impair viral replication.Through utilising flow cytometry we reveal that VZV targets immature and surface MR1 expression in MR1 overexpressing epithelial cell lines. Transfection assays identified VZV encoded serine/threonine kinase open reading frame (ORF) 66 as mediator of MR1 downregulation. We also assessed the capacity of VZV to infect primary blood MAIT cells. Flow cytometric analysis revealed that VZV infects MAIT cells at a comparable rate to conventional T cells, whilst also retaining a highly expressed extravasation and skin homing program. Finally, through spectral cytometry and *in vitro* MAIT cell stimulation assays, we demonstrated a striking abrogation of cytokine production and cytolytic capacity by VZV infected (viral antigen positive) and VZV exposed (viral antigen negative) MAIT cells in response to distinct modalities of activation such as TCR-dependent and cytokine driven activation. Overall the work from this thesis reveals a previously unknown capacity for VZV to disarm the host MR1-MAIT cell axis.

Table of contents

Statement of originality ii
Authorship attribution statement....iii
Additional publications arising during candidature....v
Presentations from this study vi
Dedication and acknowledgements vii
Abstract....ix
Contents x
Definitions....xii
List of figures and tables xiv
Introductory statement.... 1
Chapter 1 2
1.1 Herpesviruses and alphaherpesviruses.... 2
1.1.1 VZV replication cycle and spread 3
1.1.2 VZV pathogenesis 6
1.2 The immune response to VZV 12
1.2.1 Innate immune response during varicella infection 12
1.2.2 Adaptive immune response during varicella infection.... 14
1.3 The race for survival: VZV manipulation of the host immune response 16
1.3.1 Playing hide and seek: VZV impairment of host detection systems 16
1.3.2 Infect and disarm: VZV lymphotropism and functional consequences 20
1.4 MR1: A sensor for homeostatic metabolism.... 22
1.4.1 Form and function: MR1 structure and ligand binding domain 23
1.4.2 MR1 ligand repertoire and trafficking pathway 25
1.5 Sentinels of the mucosa: MAIT cells 30
1.5.1 MAIT cell development and localisation 31
1.5.2 Primed for response: The resting MAIT cell phenotype 32
1.5.3 MAIT cell activation 36
1.6 MAIT-TCR driven antimicrobial and homeostatic responses.... 40
1.7 MAIT cells in viral diseases 42
1.7.1 MAIT cells during *invitro* and acute viral infection settings.... 43
1.7.2 MAIT cells in chronic viral settings 45
1.8 Master manipulators: Herpesvirus interactions with the MR1-MAIT cell axis .. 49
1.9 Concluding remarks, hypothesis and project aims.... 51
2.1 Introductory statement 52

Chapter 3. Infection of Mucosal Associated Invariant T cells by Varicella Zoster Virus 65
3.1 Introductory statement 65
Chapter 4. Functional impairment of Mucosal Associated Invariant T cells by Varicella Zoster Virus 83
4.1 Introductory statement 83
Chapter 5. Discussion 109
5.1 Introductory statement 109
5.2 VZV modulation of MR1 (Chapter 2) 111
5.3 VZV infection of MAIT cells (Chapter 3) 115
5.4 Functional impairment of MAIT cells during VZV infection (Chapter 4) 118
Final statement 122
Chapter 6. References 123

Definitions

5-A-RU	5-amino-6-D-ribitylaminouracil
5-OP-RU	5-(2-oxo-propylideneamino)-6-D-ribitylaminouracil
Ac-6-FP	acetyl-6-formylpterin
Aa	amino acid
AKT	protein kinase B
APC	antigen presenting cell
ARPE-19	adult retinal pigment epithelial cell line-19
ATCC	american type culture collection
β2M	beta-2-microglobulin
CCR	CC chemokine receptor
CD	cluster of differentiation
CEBPδ	CCAAT enhancer binding protein delta
CLA	cutaneous lymphocyte antigen
CNS	central nervous system
COVID-19	coronavirus disease 2019
CSV	comma separated values
CTL	cytotoxic T lymphocytes
CTV	cell trace violet
CPE	cytopathic effect
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle's medium
DP	double positive
dsDNA	double stranded DNA
E	early
EBV	Epstein-Barr Virus
E.coli	*Escherichia coli*
EOMES	eomesodermin
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
EndoH	endoglycosidase H
ER	endoplasmic reticulum
ERAD	ER-associated degradation
GFP	green fluorescent protein
GzB	granzyme B
h.p.i	hours post inoculation
HCMV	human cytomegalovirus
HEK	human embryonic kidney
HHV	human herpesvirus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HS	human serum
HSV-1	human simplex virus type 1
HSV-2	human simplex virus type 2
ICA	infectious centre assay
IE	immediate early
IFA	immunofluorescence assay
IFN	interferon

Ig	immunoglobulin
IL	interleukin
iNKT	invariant natural killer T
IRF	interferon regulatory factor
ISG	interferon stimulated genes
KSHV	Kaposi's sarcoma-associated herpesvirus
MAIT	mucosal associated invariant T
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MR1	major histocompatibility complex, class-1 related
MR1T	MR1-restricted T
NF-κb	nuclear factor-κb
NK	natural killer
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PLC	peptide loading complex
PLZF	promyelocytic leukaemia zinc-finger protein
RNA	ribonucleic acid
RORγt	retinoic acid-related orphan receptor gamma t
RPMI	Roswell Park Memorial Institute
SEM	standard error of the mean
SPICE	simplified presentation of incredibly complex evaluations
SVV	simian varicella virus
T-bet	T box transcription factor 21
TAP	transporter associated with antigen processing
TABPR	tapasin binding protein
TCR	t cell receptor
TLR	toll-like receptor
TPN	tapasin
TNF	tumour necrosis factor
UPR	unfolded protein response
VZV	varicella zoster virus
ZAP	zinc finger antiviral protein

List of figures and tables

Chapter 1

Figure 1.	VZV replication kinetics	6
Figure 2.	Pathogenesis of Varicella infection	11
Figure 3.	MR1 structure	24
Figure 4.	MR1 presentation “on-demand”	26
Figure 5.	MAIT cells are primed for polyfunctional responses	33
Figure 6.	MAIT cell activation modalities	36
Figure 7.	MAIT cells in viral diseases	44

Chapter 2

Figure 1.	VZV downregulates surface MR1 at 20 and 44 hpi	54
Figure 2.	Ligand pretreatment protects MR1 during VZV infection at 20 and 44 hpi	55
Figure 3.	VZV downregulates total MR1–GFP at 20 and 44 hpi	56
Figure 4.	Mature Endo H resistant MR1 is retained during VZV infection	57
Figure 5.	VZV ORF 66 transient expression downregulates surface MR1	58
Figure 6.	VZV rOka and rOka 66s similarly downregulate surface and total MR1 at 44 hpi	59
Supplementary figure 1.	Flow cytometry gating strategy to evaluate VZV infected target cells	63

Chapter 3

Figure 1.	VZV infects MAIT cells from human peripheral blood	68
Figure 2.	VZV infects diverse MAIT cell subsets	70
Figure 3.	VZV infection of MAIT cells is associated with expression of early activation and proliferation markers	71
Figure 4.	VZV infection of MAIT cells does not suppress CCR2, CCR5 and CCR6 expression	72
Figure 5.	VZV infection of MAIT cells retains expression of CLA and CCR4 skin homing chemokine receptor expression	73
Supplementary figure 1.		74
Supplementary figure 2.		78
Supplementary figure 3.		79
Supplementary figure 4.		80

Chapter 4

Figure 1.	VZV impairs MAIT cell activation	85
Figure 2.	VZV co-cultured MAIT cells are functionally refractory to both TCR dependent and cytokine driven stimulation	87
Figure 3.	Differential expression of transcription factors in unstimulated and stimulated VZV and mock co-cultured MAIT cells	89
Figure 4.	VZV impairment of MAIT cells is contact dependent and not mediated through soluble factors	90
Figure 5.	VZV impairs MAIT cell cytolytic potential towards bacterially stimulated target cells	91
Supplementary figure 1.	MAIT cell frequency and rate of infection across treatment conditions	103
Supplementary figure 2.	VZV exposed or infected MAIT cells do not exhibit significantly greater levels of apoptosis	104
Supplementary figure 3.	Most gE:gI negative MAIT cells remain gE:gI negative	105
Supplementary figure 4.	VZV impairs MAIT cell response across several infectious dose ratios	106
Supplementary figure 5.	VZV impairs MAIT cell polyfunctional response	107

Chapter 5

Figure 1.	Proposed model of VZV suppression of the MR1-MAIT cell axis	109

Introductory statement

Human herpesviruses have co-evolved with their hosts for an extensive period of time. This prolonged co-speciation has driven human herpesviruses to be exquisitely adapted in countering host defences and establishing a life-long latency within the host. In this thesis, we focused on Varicella Zoster Virus (VZV), which is a globally disseminated and medically significant herpesvirus pathogen that causes varicella (chickenpox) during primary infection, establishes lifelong latency and can reactivate as shingles (zoster). A key contributor to the success of VZV as a pathogen is it's profound ability to evade immune detection, whilst also productively infecting and impairing immune effector subsets. So far, the studies describing VZV interactions with the host immune response has focused on classical anti-viral components such as modulation of interferons, natural killer cells, and T cells. Recently, there has been an emergence in our understanding of non-conventional immune cell populations known as innate-like T cells which respond to broadly conserved non-protein based antigenic patterns. Of the innate-like T cell group, Mucosal Associated Invariant T (MAIT) cells are the most abundantly present population and are enriched across barrier sites and mucosal interfaces of the host. MAIT cells rapidly respond to microbial invasion or translocation across barrier sites through T cell receptor dependent recognition of riboflavin synthesis; a metabolic pathway that is conserved by diverse bacterial and fungal pathogens and presented by the antigen presenting: Major Histocompatibility Complex class-1 related gene protein (MR)1. Whilst the MR1-MAIT cell axis plays a critical role against several pathogens, our understanding of how pathogens evade this axis remains germinal. Therefore, within this thesis we sought out to investigate whether a putative master manipulator of the host immune response such as VZV can modulate the MR1-MAIT cell axis.

Chapter 1

1.1 Herpesviruses and alphaherpesviruses

Herpesviridae are pervasive across the animal kingdom with over 200 documented viruses; of which nine infect humans (Kawaguchi et al., 2018). Herpesviruses are characterised as large double stranded (ds)DNA viruses that undergo distinct phases of infection: primary infection, latency and reactivation. Herpesviruses are classified into three subfamilies: *Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae*, on the basis of their biological characteristics involving host range, replication time and cell tropism for latency. Human *Alphaherpesvirinae* comprising of Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2), and Varicella Zoster Virus (VZV) are characterised by a short replicative cycle, and the establishment of latency within sensory neurons. Human *Betaherpesvirinae* including Human Cytomegalovirus (HCMV), Human Herpesvirus 6 (HHV-6) and 7 (HHV-7) have a comparatively slower replicative cycle and establish latency within lymphoreticular tissue, salivary glands, and haematapoetic myeloid progenitor cells (Kondo and Yamanishi, 2007, Goodrum, 2016, Goodrum et al., 2004, Crawford, 2023). The human *Gammaherpesvirinae* consisting of Epstein-Barr virus (EBV) and Kaposi's Sarcoma-Associated Virus (KSHV) display the slowest replicative cycle amongst the herpesvirus subfamilies, exclusively infect lymphocytes and establish latency within lymphoid tissues and peripheral lymphocytes (Lieberman et al., 2007).

All three human alphaherpesviruses infect mucosal and skin surfaces during primary infection before establishment of latency in the sensory neurons. HSV-1 and HSV-2 reactivation commonly presents as peri-oral and genital lesions respectively (Arduino and Porter, 2008, Whitley and Roizman, 2001), whilst, VZV reactivation results in a

dermatomally restricted rash (Gershon et al., 2015). Both primary infection and the reactivation phase allows for transmission of infectious alphaherpesviruses to susceptible individuals. There is a high degree of genome conservation amongst the alphaherpesvirus, especially for genes involved in DNA metabolism and lytic replication (Cohen, 2010, Arvin and Gilden, 2013). Indeed, 65 of the 71 known open reading frames (ORF) encoded by VZV are homologous to HSV-1 (Arvin and Gilden, 2013, Baines and Pellett, 2007). This degree of homology allows for comparative studies regarding the functions of alphaherpesvirus genes, including their interaction with the host immune response.

1.1.1 VZV replication cycle and spread

VZV initially attaches to host cells begins via electrostatic interactions potentially mediated by viral glycoprotein B (gB) and heparan sulfate proteoglycans present on host cell surfaces (Zhu et al., 1995, Laquerre et al., 1998, Shukla and Spear, 2001). This is followed by non-reversible fusion of VZV glycoproteins such as gB, gH, and gL which form the core fusion complex mediating host cell entry (Connolly et al., 2011). Several host receptors including heparan sulfate (Jacquet et al., 1998), mannose-6-phophate receptor (Chen et al., 2004), myelin-associated glycoprotein receptor (Suenaga et al., 2010) and insulin degrading enzyme receptor (Li et al., 2006) are proposed to bind to the VZV core fusion complex. Recent work has also demonstrated a pivotal role for host encoded Siglec-7 binding with gB as a mediator for VZV entry into monocytes (Suenaga et al., 2022a, Suenaga et al., 2022b). It is important to note that steps within the viral replication cycle such as viral entry, viral gene expression, virus protein assembly and virion packaging and egress triggers several cellular responses (Carty et al., 2021). Herpesviruses such as VZV follow a coordinated gene expression program with distinct temporal cascades that that sequentially create an

environment favourable for viral replication (Figure 1) (Reichelt et al., 2009, Perera et al., 1992).

As indicated by the name, VZV Immediate Early (IE) proteins ORF4, 61, 62 and 63 are expressed within the first few hours after cellular entry with the assistance of host cellular machinery (Braspenning et al., 2020, Defechereux et al., 1993, Kost et al., 1995, Moriuchi et al., 1993, Reichelt et al., 2009). These viral proteins are transcriptional transactivators that directly regulate the expression of subsequent viral gene products (Sato et al., 2003, Braspenning et al., 2020). In parallel, several of the VZV IE proteins also partake in blocking host transcription factors that are critical in regulating host immune responses to viral infection. In particular, ORF61 interferes with nuclear translocation of transcription factor: nuclear factor kappa-light chain enhancer of activated B-cells (NF-κB), which is central to a plethora of anti-viral responses mounted by the host cell (Whitmer et al., 2015, Sloan et al., 2012). Whilst both ORF61 and IE62 proteins also block the production of anti-viral cytokines by targetting intereferon regululatory factor (IRF) 3 activity (Zhu et al., 2011, Sen et al., 2010).

Approximately 4-7 hours after cellular entry, the accumulation of IE proteins allows for the expression of Early (E) genes such as ORF16 (viral DNA polymerase processivity factor), ORF28 (DNA polymerase large subunit), ORF51 (origin binding factor) which are involved in viral DNA replication (Reichelt et al., 2009, Cohen, 2010, Braspenning et al., 2020). Along with viral DNA expression machinery, VZV E gene products such as ORF47 and ORF66 are also expressed (Cohen, 2010, Roizman, 2013). ORF47 and ORF66 are serine threonine kinases that phosphorylate a variety of both viral as

well as host proteins (Kenyon and Grose, 2010, Moffat et al., 2004, Erazo and Kinchington, 2010, Stevenson et al., 1994). Importantly, ORF47 can also interfere with activation of IRF3, thus abrogating the expression of several pro-inflammatory mediators (Vandevenne et al., 2011), whilst ORF66 modulates the host peptide-antigen presenting capacity through downregulating classical Major Histocompatibility complex (MHC) class I expression (Abendroth et al., 2001a, Eisfeld et al., 2007b). Furthermore, ORF66 also disables the functionality of a key anti-viral cytokine interferon (IFN)-gamma (IFN-γ), through targeting IFN signalling pathways (Schaap et al., 2005). A majority of VZV genes are either Leaky Late (LL) or Late (L) genes and are involved in the assembly of progeny virions as well as encoding structural components of the virion itself (Cohen, 2010, Mettenleiter, 2002, Braspenning et al., 2020). The full replication cycle of VZV takes approximately 12 hours with mature virions detected at 9-12hpi (Reichelt et al., 2009).

Depending on the cell type, VZV can spread cell-cell through several mechanisms, such as inducing partial fusion and subsequent pore formation of the plasma membrane between cells or fusing cells together to create a large cell known as syncytia; hallmark of VZV replication in cell culture (Reichelt et al., 2009, Rodriguez et al., 1993). Alternatively, VZV can persist on the infected cell plasma membrane and enter adjacent epithelial cells via tight junctions without the requirement for cell fusion (Reichelt et al., 2009). Furthermore, VZV enters neurons through viral fusion with the neuronal plasma cell membrane to gain access to axons before hijacking the actin cytoskeleton and retrograde transport virions to neuronal cell bodies and enter latency (Grigoryan et al., 2012, Grigoryan et al., 2015). Furthermore, syncytial formation between neurons and satellite cells allows for further viral access and infection of

neurons (Reichelt et al., 2008). Overall, VZV gene expression follows distinct temporal phases that culminate in a rapid replication cycle and a highly cell-associated spread of progeny virus.

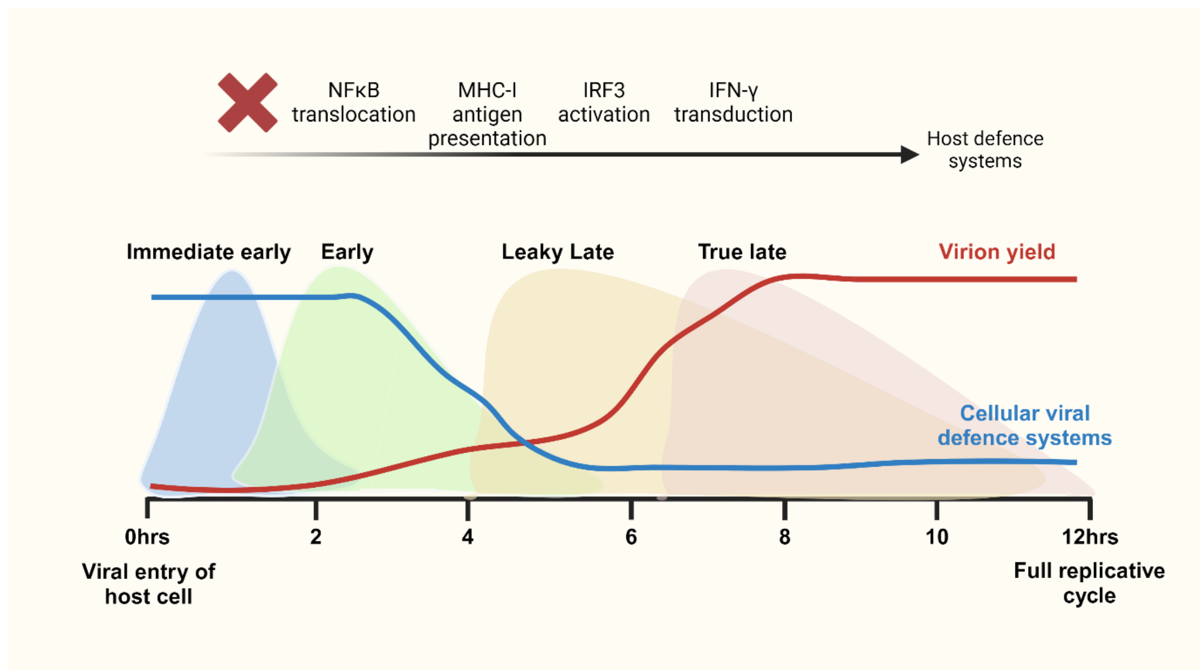

Figure 1. VZV replication kinetics
Expression of VZV genes occurs in a ordered cascade. Immediate Early genes are detected within 2 hours of cellular entry and directly initiate and regulate expression of Early genes. They also impair host sensing of viral entry and replication through disrupting NFκB pathway. This is followed by Early gene expression which further subverts host defence mechanisms such as viral peptide antigen presentation, IFN-γ signalling and IRF3 mediated cytokine expression. This allows for Leaky Late and Late gene expression. Full replication cycle takes 12 hours. Red line indicates rise in yield of virions whilst the blue line indicates decrease in cellular viral defence systems over the course of VZV replication cycle. The red cross indicates viral inhibition of host defences over the course of replication cycle as stated.
Figure created using Biorender.

1.1.2 VZV pathogenesis

Characteristic of all herpesvirus infections, VZV follows three distinct phases of infection: primary infection (varicella), latency, and reactivation (herpes zoster). Initial transmission of VZV can occur through direct contact with or inhalation of aerosolised

VZV from lesions, as well as through inhalation of infectious respiratory droplets (Tellier et al., 2019, Gershon and Gershon, 2013, Tsolia et al., 1990, Seward et al., 2004). Following initial infection of epithelial mucosa within the upper respiratory tract, it is postulated that residing intra-epithelial dendritic cells (DCs) take up and traffic the virus to local draining lymph nodes such as the tonsils (Sawyer et al., 1992, Levin, 2014) . The inherently interactive nature of DCs through direct cellular contact with other diverse immune cell subsets allows for the transmission of VZV to tonsillar T cells (Figure 2) (Morrow et al., 2003, Abendroth et al., 2001b). Here, VZV preferentially infects as well as remodels T cells to upregulate activation marker CD69, skin homing markers CCR4 and cutaneous lymphocyte antigen (CLA) (Sen et al., 2014b, Ku et al., 2002, Ku et al., 2004).

Our laboratory has demonstrated that VZV can also productively infect and upregulate skin homing capacity of circulating Natural Killer (NK) cells (Campbell et al., 2018). Lymphotropism is a critical step in dissemination of VZV throughout the host (as reviewed further in Section 1.3.2), with several studies identifying the presence of VZV DNA in peripheral blood mononuclear cells (PBMCs) such as $CD4^+$, $CD8^+$, B cells and NK cells during varicella infection (Sawyer et al., 1992, Ozaki et al., 1994, Mainka et al., 1998, Ito et al., 2001, Vossen et al., 2005b). However, it was not known whether VZV can productively infect unconventional immune cell populations and harness their endogenous predilection for skin homing. This question formed an investigative focus of this thesis.

Viremia is followed by a prolonged incubation period of 10-21 days during which the host demonstrates no symptoms (Gershon and Gershon, 2013). This protracted

incubation period is posited to be a result of VZV evading and overcoming several host defence responses (Abendroth and Arvin, 2001, Abendroth et al., 2010). Following the incubation period, the host presents with highly contagious vesiculopustular lesions disseminated across the cutaneous sites as well as mucous membranes such as the oral cavity; known as varicella (chicken pox) (Gold, 1966, Tsolia et al., 1990, Gershon and Gershon, 2013, Arvin and Gilden, 2013).

At the skin, VZV infects epithelial cells, keratinocytes and dermal fibroblasts (Tommasi and Breuer, 2022, Taylor and Moffat, 2005, Arvin et al., 2010), as well as skin resident dendritic cell populations such as Langerhan cells and infiltrating plasmacytoid DCs (Huch et al., 2010). Importantly, the manifestation of systemic cutaneous vescicular lesions disrupts the normal skin architecture which consequently allows for translocation of commensal microbes. This is clinically observed with bacterial superinfections such as bacterial cellulitis to pneumonia and/or sepsis reported as the most common complications arising from severe VZV infection (Diniz et al., 2018, Ziebold et al., 2001, Gershon and Gershon, 2013). It is not known what the impact of invading microbiota during varicella infection has on the activation of skin residing innate and adaptive immune subsets sensitive to microbial presence. In Section 1.5 we cover a mucosal enriched unconventional immune axis that detects pathogen invasion through responding to riboflavin biosynthesis; a metabolic pathway that is not present in mammals but conserved across diverse microbiota.

It takes approximately 1-2 weeks for the host to resolve varicella infection, during which time VZV is able to infect sensory neurons in dorsal root ganglia (DRG) through retrograde axonal transport (Gilden et al., 1983, Gilden et al., 2015, Markus et al., 2011, Grigoryan et al., 2012, Grigoryan et al., 2015). Alternatively, VZV is able to

access the DRG through hematogenous transport via infiltrating immune cells (Zerboni et al., 2005, Ouwendijk et al., 2013, Gilden et al., 2015) (Figure 2). Interestingly, VZV can also access and establish latency in the enteric nervous system; therefore explaining gastrointestinal discomfort that sometimes accompanies VZV infection (Chen et al., 2011). Within the DRG, VZV establishes a lifelong latency within the host, which is characterised through the expression of a limited set of viral genes which are VZV associated latency transcript (VLT) and ORF63 (Depledge et al., 2018b). VLT is an anti-sense sequence to ORF61 and functions as a suppressor of ORF61 gene transcription (Depledge et al., 2018a), whilst expression ORF63 RNA is also is critical for the establishment of VZV latency (Cohen et al., 2004). Strikingly, human induced pluripotent stem cell (iPSC)-derived sensory neurons (HSN) based *in vitro* models of VZV reactivation from latency have demonstrated that the presence of VLT-ORF63 fusion transcripts precede VZV lytic gene expression and therefore hypothesised to drive VZV reactivation (Ouwendijk et al., 2020).

It has been postulated that VZV reactivates multiple times during a host's lifetime, however does not usually progress to clinical disease (Freer and Pistello, 2018). However, VZV-specific immunity can wane either naturally through age associated immunosenenesence, or induced following viral immunosuppression (eg. HIV infection) (Gottlieb et al., 1983) or through immune-suppressive medical treatments for transplant and cancer patients (Locksley et al., 1985, Hayes and Feldman, 1978). VZV reactivates along a single dermatome and presents as a unilateral rash known as herpes zoster (shingles) (Hope-Simpson, 1965, Muraki et al., 1992). Interestingly, VZV DNA can be detected in the blood of immune-compromised patients prior to the onset of the herpes zoster rash (Guiraud et al., 2023). Furthermore, viremia can persist for

several months in healthy patients after the resolution of the herpes zoster rash (Quinlivan et al., 2011, Levin, 2014).

Post-herpetic neuralgia (PHN) is frequently a severely debilitating neuropathic pain syndrome associated with 5-30% of herpes zoster patients (Mallick-Searle et al., 2016, Kawai et al., 2014, Rogers III and Tindall, 1971) and can persist for months to years after resolution of herpes zoster rash. Currently, the aetiology of neuropathic pain following zoster remains unknown, and therefore the treatment of PHN is largely limited to pain mitigation instead of curative treatments (Tang et al., 2023, Niemeyer et al., 2024b). The prolonged nature of PHN combined with inadequate treatment options therefore places a substantial burden on both the patient as well as the healthcare system (Friesen et al., 2017, Friesen et al., 2016, Pan et al., 2022).

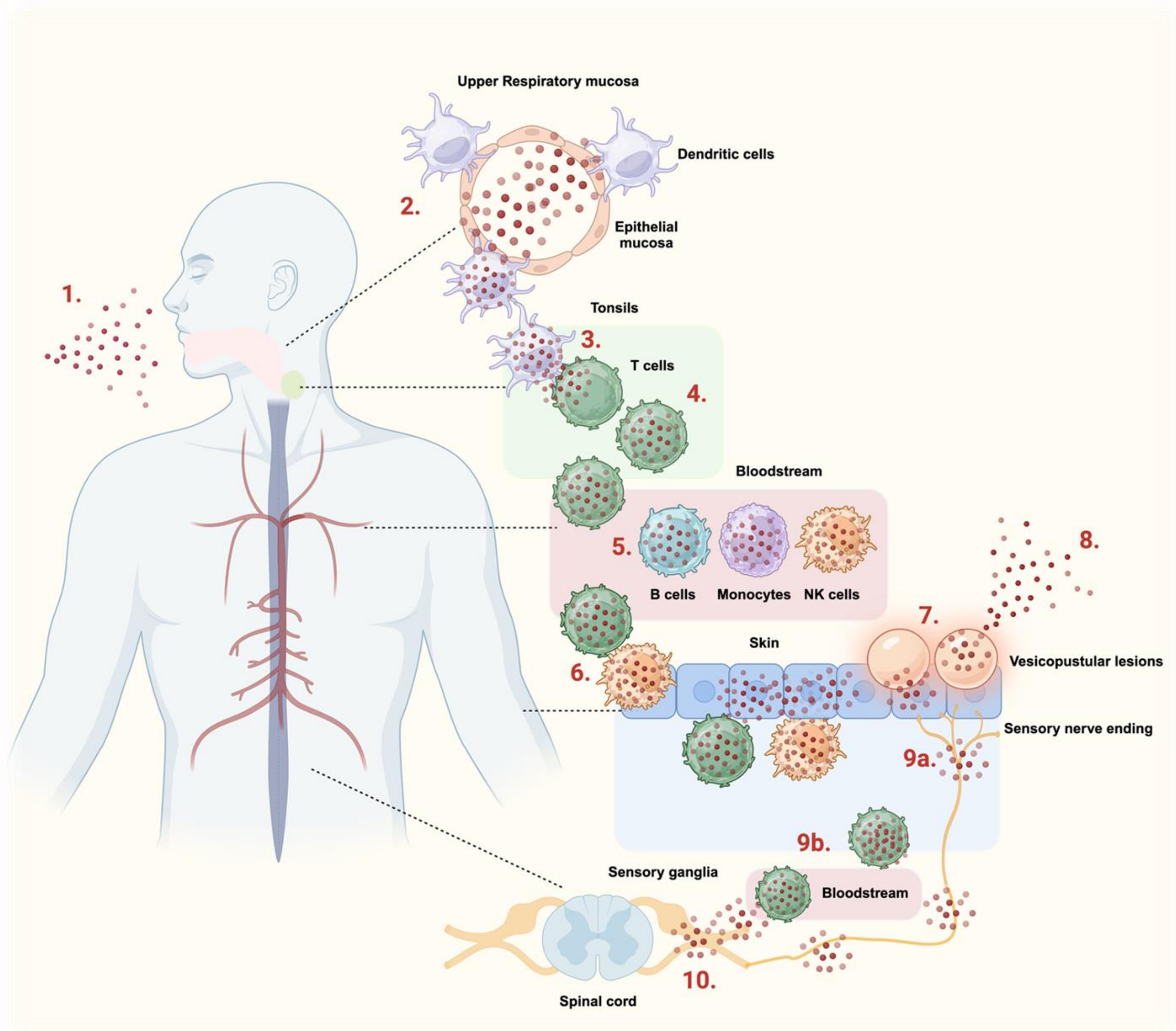

Figure 2. Pathogenesis of Varicella infection
Inhalation of VZV infected aerosols (1) results in the initial infection of upper respiratory mucosa and intra-epithelial dendritic cells (DC) (2). Drainage of infected DCs to local tonsillar lymph nodes (3 and subsequent infection of tonsillar T cells (4). VZV also infects circulating B cells, monocytes and NK cells(5). VZV infected T cells and NK cells transport virus to skin sites (6), resulting in infection of keratinocytes presenting as in vesiculopustular lesions (7). Cell free virions can be released from pustules to transmit virus to next host (8). Retrograde axonal transport through skin innervating nerves (9a) or T cell associated hematogenous spread (9b) allows VZV to access the sensory ganglia and establish life-long latency (10).
Figure created using Biorender.

1.2 The immune response to VZV

The concerted generation of a robust innate and adaptive immune response by the host during varicella is required for the resolution of infection. This is underscored by severe or fatal outcomes to varicella infections for patients with selective immune-deficiencies within innate or adaptive arms of the immune response (Ansari et al., 2021).

1.2.1 Innate immune response during varicella infection

VZV is non-specifically detected through several pattern recognition receptors (PRRs) designed to recognise viral associated molecular patterns. Cell surface Toll-like receptors (TLR) 2 recognises VZV glycoproteins (Wang et al., 2005) whilst endosomal TLR3 and TLR9 detects viral dsDNA (Yu et al., 2011, Sironi et al., 2017). The triggering of TLRs results in the downstream activation of transcription factors such as NF-κB, IRF3 and IRF7 which induces the expression of several type 1 interferons and pro-inflammatory cytokines which create an “anti-viral” environment that limits viral replication and spread (Paludan et al., 2011). The importance of establishing an anti-viral state during varicella infection through TLR detection is underscored by reports of severe viral encephalitis in patients with TLR3 deficiency (Sironi et al., 2017).

Through TLR2 triggering, monocytes and monocyte derived macrophages produce pro-inflammatory cytokines such as interleukin (IL)-6, IL-8 and tumour necrosis factor alpha (TNF-α) in response to VZV infection *in vitro* (Wang et al., 2005). Production of TNF-α is critical for controlling viral replication (Ito et al., 1991), with patients undergoing anti-TNF-α treatment reporting increased VZV reactivation (Cacciapaglia et al., 2015). Additionally, IFN-α plays a key role in limiting VZV replication with reports

correlating reduced serum IFN-α levels with increased varicella severity (Arvin et al., 1986). This is further recapitulated with a reduction of varicella severity in immune-compromised children through IFN-α treatment (Arvin et al., 1982). Plasmacytoid DCs (pDCs) are likely a key contributor as they produce abundant IFN-α through TLR9 triggering (Yu et al., 2011). Interestingly, elevated IFN-α is present in serum of healthy patients prior to and during acute phase of varicella, with reduced expression correlating with poorer disease outcomes (Arvin et al., 1986). Whilst IFN-α is critical in delaying and limiting infection, IFN-γ has been demonstrated to more potently inhibit VZV replication (Sen et al., 2018). NK cells are prolific producers of IFN-γ as well as mediators of direct cytolysis of VZV infected cells through granulysin and granzyme B expression (Hata et al., 2001, Ihara et al., 1989). Indeed, the protective role played by NK cells during varicella infection is highlighted by severe or fatal outcomes observed in patients with either NK cell deficiencies and/or reductions (Biron et al., 1989, Etzioni et al., 2005, Notarangelo and Mazzolari, 2006, Mace et al., 2013, Vossen et al., 2005a).

At the skin, IFN-α production is critical in limiting infection to localised skin sites (Ku et al., 2004, Arvin et al., 1982). This is consistent with high expression of IFN-α by epidermal epithelial cells (Ku et al., 2004) as well as corresponding with a marked influx of pDCs (Huch et al., 2010). It is not clear what role skin resident innate-like T cell populations such as Mucosal Associated Invariant T (MAIT) cells play during varicella infection given their propensity to abundantly produce pro-inflammatory mediators in response to cytokine stimulation. Altogether, the secretion of key pro-inflammatory cytokines such as IFN-α, IFN-γ, and TNF-α are critical in both controlling

viral replication to a localised setting whilst also providing time for the host to mount a polarised adaptive immune response.

1.2.2 Adaptive immune response during varicella infection

Approximately 90% of the human population is seropositive for VZV, with immunoglobin (Ig)G antibodies detected for a range of VZV glycoproteins and tegument proteins (Ceroni et al., 2010). However, neither the magnitude of antibody production or B-cell deficiencies are correlated with varicella severity; suggesting a limited role for humoral immunity during infection (Arvin et al., 1986, Palumbo et al., 1984, Webster et al., 1989). In contrast, the induction of an early T cell mediated immune response strongly correlates with effective control of varicella (Arvin et al., 1986, Malavige et al., 2008). VZV specific $CD4^{+}$ and $CD8^{+}$ T cells are detectable approximately three days after the onset of the varicella rash (Malavige et al., 2008, Arvin et al., 1986).

Circulating $CD4^{+}$ T cells with an activated, skin homing profile are detected during early varicella, and function to clear VZV from the skin (Malavige et al., 2008). These T cells correlate with viral clearance (Vossen et al., 2004), and are independent of skin trafficking $CD4^{+}$ T cells utilised by VZV for dissemination to cutaneous sites (Sen et al., 2014b). Abundant levels of IFN-α in serum and at the skin polarises $CD4^{+}$ T cells to a T helper type 1 (Th1) response (Yu et al., 2005). Th1 $CD4^{+}$ T cells secrete IFN-γ and IL-2, which further propagates an anti-viral state and also licences $CD8^{+}$ T cell driven cytotoxic response (Jenkins et al., 1998). Interestingly, $CD4^{+}$ T cells can also directly participate in the killing of VZV infected cells through MHC class II recognition (Cooper et al., 1988, Diaz et al., 1989). Within varicella lesions, $CD8^{+}$ T cells

expressing cytolytic enzyme granzyme B can be detected (Morizane et al., 2005), whilst *in vitro* studies have demonstrated granulysin production by $CD8^+$ T cells effectively kills VZV infected cells (Hata et al., 2001). Furthermore, granzyme B can also additionally control VZV infection through selectively cleaving IE viral proteins such as ORF4 and ORF62 (Gerada et al., 2019). The antigenic repertoire of $CD4^+$ and $CD8^+$ T cell responses encompasses VZV proteins derived from envelope, tegument, glycoproteins (Arvin et al., 1986, Diaz et al., 1989, Huang et al., 1992, Sharp et al., 1992, Sadzot-Delvaux et al., 1997) and maintains a long lasting persistence within the host (Ogunjimi et al., 2014). The importance in retaining a lasting $CD4^+$ T cell response is clinically observed through reports of severe varicella or increased recurrent VZV reactivation in patients with either an age related or HIV driven $CD4^+$ T cell decrease (Weinberg and Levin, 2010, Weinberg et al., 2009, Gershon et al., 1997). Overall, VZV specific T cells are required for the resolution of varicella and control the capacity for VZV reactivation during the the host's lifetime.

Interestingly, invariant natural killer T (iNKT) cell deficiency is associated with live attenuated vaccine derived severe disseminated varicella infection (Levy et al., 2003, Banovic et al., 2011). However, iNKT cells respond to lipid antigens presented by non-classical MHC class I-like CD1d molecules (Bendelac et al., 1995); of which no viral ligands have been described. This raises the intriguing possibility that other non-viral antigen based unconventional immune cell axes may also potentially play a critical role in the control of VZV infection.

1.3 The race for survival: VZV manipulation of the host immune response

Following initial entry into the host, the invading pathogen faces a temporally sensitive selective pressure to establish infection before the host mounts a defence response that eradicates the pathogen. Despite the generation of a robust and long-lasting immune response, VZV is able to successfully infect and establish a life-long latent infection within the host. This suggests active evasion and modulation of host defences systems during early VZV infection which provides a transient window for the establishment of infection. Indeed, through disrupting several arms of host immune surveillance systems as well as infection and functional manipulation of immune subsets; VZV profoundly evades immune recognition and suppresses corresponding effector response. Here, we focus on how VZV modulates the classical anti-viral host immune axes such as T cell and NK cell driven immune responses.

1.3.1 Playing hide and seek: VZV impairment of host detection systems

The ability of the host to efficiently capture and present viral peptide antigens from various subcellular compartments during productive infection dictates the initiation of a cell-mediated immune response. Therefore, it is not surprising that VZV as well as other herpesviruses encode an array of strategies that differentially disrupt host peptide based recognition of viral infection.

Viral proteins derived from the cytoplasm are efficiently processed and loaded onto MHC-I within the endoplasmic reticulum (ER) with the help of several chaperone proteins that form the Peptide Loading Complex (PLC) (Cresswell et al., 1999). The loading of antigenic peptide onto MHC-I is therefore a bottleneck that herpesviruses masterfully exploit. Briefly, HSV-1 encoded ICP47 directly binds to transporter

associated with antigen processing (TAP), to corrupt loading of peptides onto MHC-I (Früh et al., 1995, Orr et al., 2005, Hill et al., 1995). Direct binding and modulation of TAP functionality is also observed by HCMV encoded US3 protein (Park et al., 2004). Additionally, HCMV impairs energy dependent TAP mediated transport of peptides into the ER by disrupting adenosine triphosphate (ATP) hydrolysis via US6 protein (Halenius et al., 2006). Curiously, VZV encoded UL49.5 protein also directly interacts with TAP, however does not disturb the capacity for peptide loading (Koppers-Lalic et al., 2008). Instead, VZV targets the secretory pathway stage by retaining MHC-I complexes within the Golgi apparatus (Abendroth et al., 2001a); this was demonstrated to be partially mediated through ORF66 (Abendroth et al., 2001a, Eisfeld et al., 2007b). Furthermore, it was demonstrated that VZV IE proteins (ORF4, IE62 and IE63) are profoundly depleted of high-affinity MHC-I epitopes (Meysman et al., 2016). The authors proposed this selective depletion to delay MHC-I detection during early stages of viral infection; therefore increasing the chance of generating viral progeny (Meysman et al., 2016). In a subsequent study, the authors revealed the significance of this phenomena by correlating the poor presentation of IE62 by human leukocyte antigen (HLA)-A with increased herpes zoster risk (Meysman et al., 2018). It is not currently known whether VZV is also able to target other antigen presenting molecules that also reside within the ER whilst awaiting ligand binding. In Chapter 2 of this thesis, we explore whether VZV exploits this bottleneck within the biosynthetic pathway of a non-classical MHC-I related molecule (MR1).

Furthermore, professional antigen presenting cells (APCs) can effectively present endosomal, lysosomal and cytoplasmic viral antigens via MHC-II. This is shown through MHC-II presentation of HCMV IE1 (Le Roy et al., 2002) and EBV nuclear

antigen (Münz et al., 2000, Voo et al., 2002). However, unlike MHC-I, MHC-II expression is inducible and regulated through Class II transactivator (CIITA) which is influenced through IFN-γ signalling via the JAK-STAT pathway (Tur et al., 2021, Beresford and Boss, 2001). Again, VZV exploits this induction through targeting STAT1 and JAK2 signalling to block downstream transcription of IRF1 and CIITA; therefore curtailing MHC-II expression (Abendroth et al., 2000). Ultimately, the modulation of antigen presentation by MHC-I and MHC-II molecules is believed to effectively restrict and evade $CD8^+$ and $CD4^+$ T cell immune-surveillance (Abendroth and Arvin, 2001).

However, the suppression of MHC-I molecules can lead to virally infected cells being potentially susceptible to NK cell mediated killing. Briefly, NK cells encode inhibitory receptors that bind to MHC-I as well as activating receptors that bind to stress induced cellular ligands (Lanier, 2005). Through loss of inhibitory receptor engagement and binding of activating receptors to stress ligands enables NK cells to mount an immune response against virally infected cells that have lost MHC-I expression (Lanier, 2005). Perhaps the most well characterised NK cell receptor: Natural Killer group 2 member D protein (NKG2D) binds to eight currently known stress-induced ligands associated with infection (Raulet, 2003). Remarkably, VZV infected epithelial cells co-cultured with primary human NK cells failed to elicit degranulation; therefore suggesting potentially lack of NKG2D mediated detection (Campbell et al., 2015). Analysis of VZV infected cells revealed differential modulation of NKG2D ligands as cell surface UL binding protein (ULBP) 2 and ULBP3 were downregulated, whilst cell surface MHC-I chain related protein A (MICA) was upregulated (Campbell et al., 2015). Intriguingly, this differential pattern of NKG2D ligand modulation was not observed in HSV-1

infection, which instead resulted in total and surface downregulation of ULBP2, ULBP3 and MICA (Campbell et al., 2015). Through exhibiting distinct patterns of NKG2D ligand modulation, both alphaherpesviruses are able to restrict the NK cell anti-viral response.

Herpesviruses have also demonstrated the capacity to target non-peptide based antigen presenting systems of which no direct viral ligands have been yet described. CD1d is loaded with endogenous ligands within the ER (De Silva et al., 2002) and requires endosomal recycling of surface CD1d to replace endogenous ligand with exogenous ligands (Chiu et al., 2002) to present to iNKT cells (Bendelac et al., 1995). Both the ER loading and endosomal recycling exchange steps therefore present bottlenecks for herpesviruses to exploit. Indeed, HCMV encoded Us2 protein binds to and directs ER resident immature CD1d to ubiquitin-dependent proteosomal degradation (Han et al., 2013). Whilst, HSV-1 through the combined actions of Us3 and gB prevent the ligand exchange step by redirecting surface bound CD1d to the TGN (Yuan et al., 2006, Rao et al., 2011, Xiong et al., 2015). Importantly, modulation of CD1d antigen presentation by HCMV and HSV-1 resulted in impairment of iNKT TCR dependent activation and functional responses (Yuan et al., 2006, Rao et al., 2011).

Recently, we demonstrated that VZV also profoundly modulates total and surface CD1d protein expression as well as CD1d transcript abundance (Traves et al., 2023) Furthermore, the downregulation of surface and total CD1d was partially mediated through VZV ORF66 (Traves et al., 2023). These findings reveal an evolutionary investment by VZV to disrupt non-peptide antigen presenting pathways that do not as-of-yet demonstrate a direct selective pressure on viral pathogenicity. It remains to be

demonstrated if VZV also disrupts other non-viral ligand presenting pathways such as MR1 which presents metabolite based antigens; we investigate this possibility in Chapter 2.

Ultimately, the modulation of several host antigen presentation pathways is likely an important pathogenic strategy employed by VZV to restrict detection from diverse immune subsets that have mount anti-viral responses.

1.3.2 Infect and disarm: VZV lymphotropism and functional consequences

It is understood that lymphotropism, particularly for T cells by VZV, is crucial to the intra-host dissemination of virus during primary infection, with extensive literature characterising this interaction and its contribution to VZV pathogenesis (Moffat et al., 1995, Moffat and Arvin, 1999, Ku et al., 2002, Ku et al., 2004, Sen et al., 2014b, Sen et al., 2015, Moffat et al., 1998). Whilst not as well characterised, VZV lymphotropism also extends to other populations such as B cells (Gutzeit et al., 2010, Jones et al., 2019),NK cells (Campbell et al., 2018, Campbell et al., 2019) as well as a variety of myeloid cells such as immature dendritic cells (DC), mature DCs (Morrow et al., 2003, Abendroth et al., 2001b), pDCs (Huch et al., 2010), undifferentiated monocytes and macrophages (Kennedy et al., 2019). Here, we will focus on the impact of VZV exposure and infection to the functionality of T cells and NK cells.

As reviewed in Section 1.1.2, VZV infection of T cells and NK cells enhances the skin homing program mediated through chemokine markers such as CLA and CCR4 (Campbell et al., 2018, Ku et al., 2002, Ku et al., 2004, Sen et al., 2014b). In addition to harnessing NK and T cells for skin trafficking, VZV must also counter the ability of NK and T cells to respond to inflammatory cues which are present both systemically

and at the skin; as reviewed in Section 1.2.1 and 1.2.2 respectively. Indeed, previous landmark studies using single mass cytometry elegantly revealed a substantial rewiring of phosphorylation networks of VZV infected tonsillar T cells within the TCR signalling cascade chain (Sen et al., 2014b). Specifically, VZV infection was associated with increased Zap70 phosphorylation; a proximal signalling event following TCR engagement that classically leads to a NF-κB mediated immune response via the ERK1/2 pathway (Yokosuka et al., 2005, Cheng et al., 2011). Surprisingly, increased Zap70 phosphorylation within VZV infected T cells instead activated non-classical pathways of cellular proliferation; therefore diverting the TCR signalling cascade away from a functional inflammatory response (Sen et al., 2014b). Whilst assessment of cytokine release by VZV infected T cells in response to stimulation remains to be explored, rewiring of the TCR signalling cascade is presumed to divert phosphorylation away from NFκB; therefore curtailing a plethora of downstream T cell mediated inflammatory responses allowing for a robust host immune response (Baldwin, 2001).

In this regard, HSV-1 encoded protein kinase Us3 partially inhibits TCR signalling through disrupting activation of key pathway protein LAT, therefore restricting IL-2 production (Yang et al., 2015). More, recently, Campbell et al., demonstrated that both VZV infected and exposed (VZV antigen negative) PBMC derived NK cells are functionally refractory to stimulation (Campbell et al., 2019). Specifically, both VZV exposed and infected NK cells exhibited impaired activation resulting in decreased IFN-γ and TNF expression in response to stimulation (Campbell et al., 2019). The functional restriction of NK cells in producing IFN-γ and TNF likely enhances VZV

pathogenesis, given the critically protective role these cytokines play during varicella infection (Ito et al., 1991, Sen et al., 2018, Torigoe et al., 2000).

Overall, VZV plays a balancing act of utilising lymphocytes for replication and transmission to skin sites whilst restricting their functional response to inflammatory stimuli. It is not known whether VZV functionally modulates other classes of immune populations such as innate-like T cells. In particular, mucosal associated invariant T (MAIT) cells are abundant in circulation and skin and copiously produce pro-inflammatory cytokines upon activation (Provine and Klenerman, 2020). We explore whether VZV can infect human MAIT cells (Chapter 3) and investigate the functional consequences of VZV interaction with MAIT cells in Chapter 4.

1.4 MR1: A sensor for homeostatic metabolism

Differentiation between foreign and self is fundamentally mediated through the binding and presentation of diverse antigens by conventional and non-conventional antigen presenting molecules. Conventional MHC-I and MHC-II molecules are highly polymorphic and therefore can present a wide array of peptide antigens to cognate conventional $CD8^{+}$ and $CD4^{+}$ T cells, respectively. Conversely, the MHC-I related molecule MR1 is a highly monomorphic molecule that is deeply conserved in expression across the mammalian spectra (Bugaut et al., 2023, Riegert et al., 1998, Tsukamoto et al., 2013). The monomorphic nature of MR1 and homologous conservation across mammals suggests an evolutionary imperative for the detection of a specific antigenic pattern that is concordantly conserved by diverse pathogens. Seminal studies have demonstrated that MR1 binds to and presents unstable metabolite derivates from riboflavin biosynthesis (Corbett et al., 2014, Kjer-Nielsen et

al., 2012); a metabolic pathway that is not present in mammals or viruses, but encoded by various bacterial and fungal species (Tastan et al., 2018, Mondot et al., 2016). Interestingly, a recent landmark study has revealed MR1 also presents a different class of host-microbiota derived metabolite antigens from digestive bile acid pathways (Ito et al., 2024). Thus, the surveillance of abundant metabolite patterns that can be host, commensal or pathogen associated therefore allows MR1 to function as a sensor for perturbations in homeostatic cellular metabolic processes (Chancellor et al., 2022).

1.4.1 Form and function: MR1 structure and ligand binding domain

MR1 shares several structural similarities to MHC-I such as signal domain, cytoplasmic domain, extracellular immunoglobin domains, a short helical transmembrane domain, as well as association with light chain beta-2 microglobulin (β2m) (Hashimoto et al., 1995). The cytoplasmic tail of MR1 contains a tyrosine residue motif (Figure 3) that is not commonly observed in other antigen presenting molecules (Lim et al., 2022) and plays a specialised role in the endocytic capacity of MR1, as reviewed in Section 1.4.2.3. It is however the core differences within the antigen binding cleft that allows for MR1 to specialise in the capturing of metabolite based antigens (Hansen et al., 2007) (Figure 3). MHC-I has a large antigen binding cleft with six side pockets (Garrett et al., 1989) that can accommodate peptides between 8-11 amino acids long (Rock et al., 1994). In comparison, the MR1 antigen binding cleft is composed of pockets A' and F' with no accompanying side pockets (Keller et al., 2017b, López-Sagaseta et al., 2013). Furthermore, the binding cleft is unfavourable for peptide binding by containing several aromatic, basic and hydrophobic residues creating an "aromatic cradle" that instead predisposes the capturing of organic metabolite products (Hansen et al., 2007, Miley et al., 2003)

(Figure 3). Critically, a charged lysine residue in position 43 (K43) within the binding groove prevents the complete folding of MR1 and instead retains MR1 in an open ligand receptive conformation within the cells endoplasmic reticulum (Chua et al., 2011, McWilliam et al., 2016b).

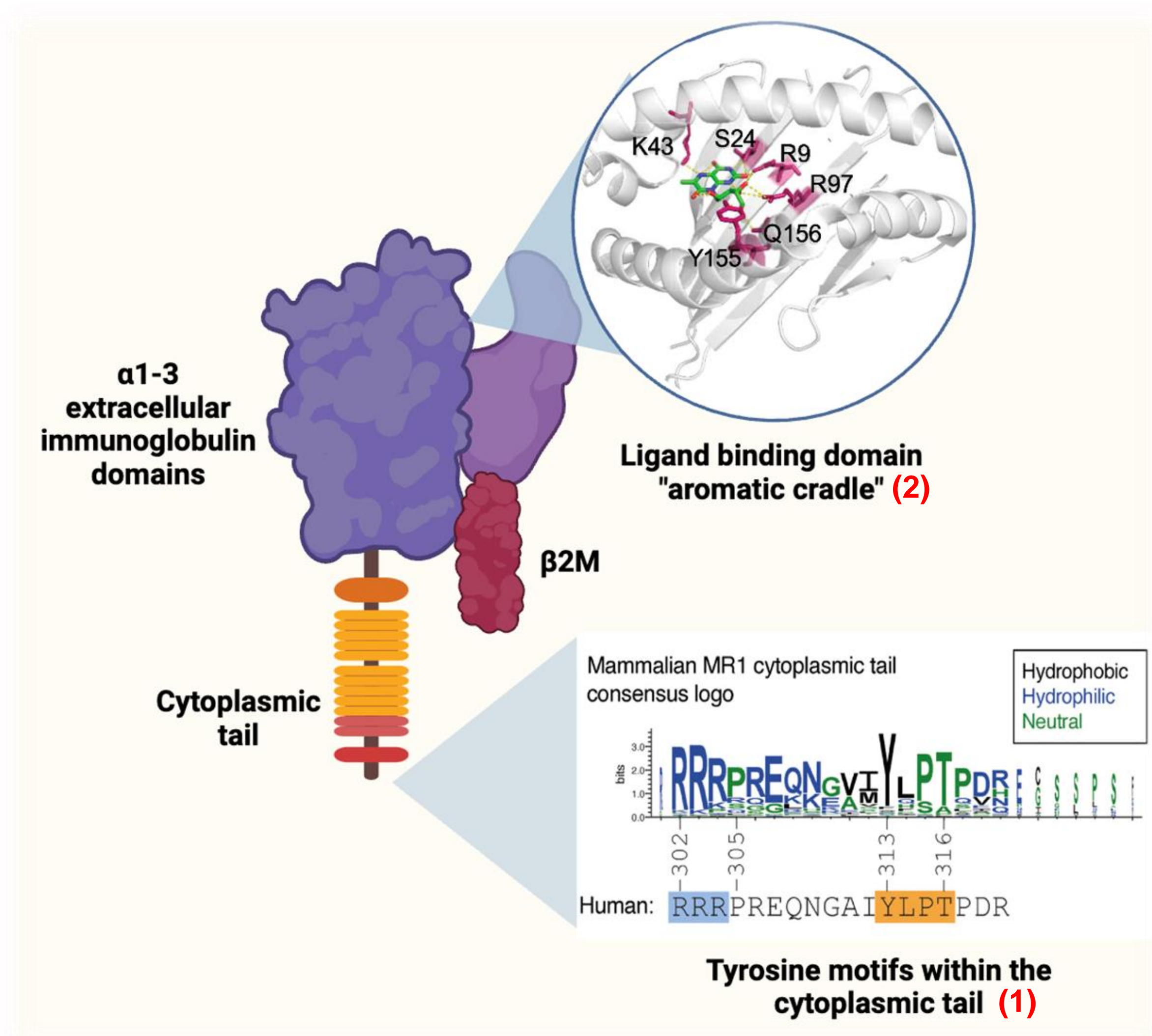

Figure 3. MR1 structure

The mature MR1 protein is composed of several distinct components such as the extracellular immunoglobulin domain, cytoplasmic tail and association to beta 2 microglobulin (β2m). In particular, the cytoplasmic tail of MR1 is enriched with a tyrosine residue (1) (Lim et al., 2022). Furthermore, the ligand binding domain is lined with several aromatic, basic and hydrophobic residues that create an "aromatic cradle" (2) (Kjer-Nielsen et al., 2012, Eckle et al., 2015). These characteristics allow MR1 to specialise in the capturing and presenting of metabolite based antigens.
(**1**) Cytoplasmic tail consensus sequence figure sourced from: (Lim et al., 2022).
(**2**) Crystal image of MR1 ligand binding domain sourced from: (Eckle et al., 2015).
Figure created using Biorender.

1.4.2 MR1 ligand repertoire and trafficking pathway

Despite presenting a molecular metabolite pattern abundant within the host, cell surface MR1 expression is highly limited (Abós et al., 2011, Chua et al., 2011); therefore indicating the surface expression of MR1 protein to be a tightly regulated process. Indeed, there are several layers of host derived regulation that control each step of the MR1 biosynthetic pathway: ligand binding, ER egress, and endocytosis (McWilliam and Villadangos, 2023) (Figure 4).

1.4.2.1 Awaiting capture: MR1 ligand binding within the ER

Within the ER, MR1 predominantly accumulates in an open conformation that does not associate with β2m (McWilliam et al., 2016b, McWilliam et al., 2020). Without the assistance of any chaperones, accumulation of empty MR1 within the ER would usually result in degradation due to it's inherent conformational instability (Römisch, 2005). However, recent studies have revealed that PLC associated member TAP and PLC independent protein Tapasin Related Protein (TABPR) stabilise nascent unloaded MR1 within the ER (McShan et al., 2022, McWilliam et al., 2020). Furthermore, the accumulation of immature MR1 is also regulated by an ER resident translocase protein known as ATP13A1 (Kulicke et al., 2022) which removes misdirected mitochondrial proteins out of the ER (McKenna et al., 2020). However, the direct manner through which ATP13A1 assists in maintaining immature MR1 is not known. Overall, several ER resident chaperone proteins play a critical role in the maintenance of ligand receptive depot of immature MR1 within the ER.

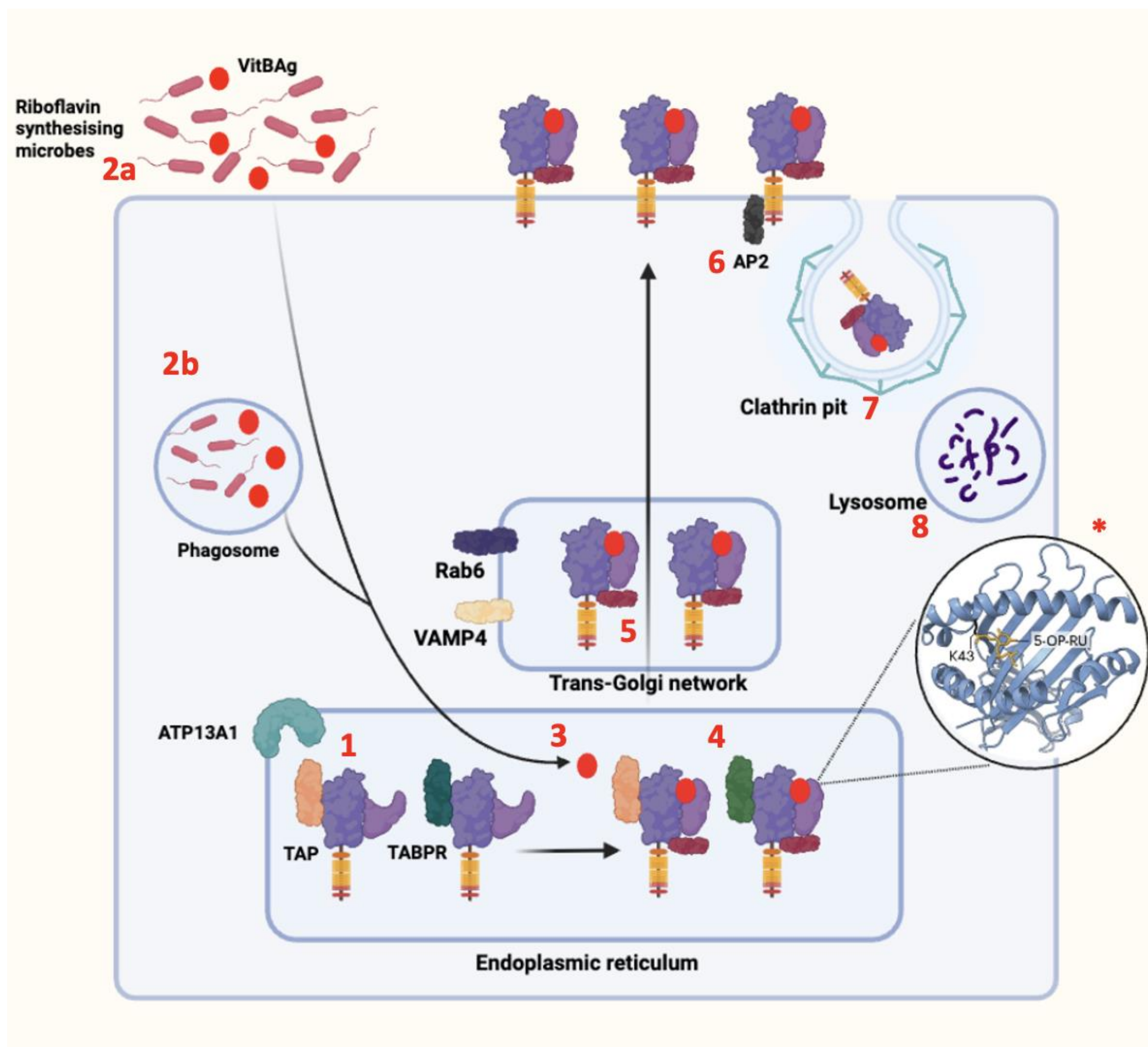

Figure 4. MR1 presentation "on-demand"
(**1**) MR1 accumulates within the endoplasmic reticulum (ER) in an immature, ligand receptive open conformation. This unstable conformation is stabilised by proteins: transporter associated with antigen processing (TAP) and TAP related protein (TABPR). Furthermore, ER resident translocase protein known as ATP13A1 also supports immature MR1 accumulation. Vitamin B antigens (VitBAgs) from extracellular microbes (**2a**), or from internalised microbes within phagasomes (**2b**) are loaded onto immature MR1 within the ER through unknown mechanisms (**3**). The binding of immature MR1 with Schiff base forming compounds such as 5-OP-RU neutralises the lysine residue (K43) within the binding cleft and triggers a molecular switch leading to closed MR1 conformation and association with beta 2 microglobulin (**4**). Ligand bound MR1 traffics to the plasma surface through the Trans-Golgi network (TGN) via the secretory pathway (**5**). TGN associated proteins RAB6 and VAMP4 assist in MR1 trafficking. Following antigen presentation, AP2 binds to the cytoplasmic tail of MR1 (**6**) resulting in Clathrin mediated endocytosis of MR1 complex (**7**). Most endocytosed MR1 complexes are degraded within lysosomes (**8**).
(*) Crystal structure image sourced from: (McWilliam and Villadangos, 2023).
Figure created using Biorender.

The retention of open conformation MR1 within the ER suggests a fundamental dependence on a class of antigens that can neutralise residue K43 through a covalent bond known as Schiff base. Seminal studies have since revealed that MR1 binds to photodegradation derivates from both the folic acid (Vitamin B9) as well as unstable precursors and intermediates from the riboflavin (Vitamin B12) biosynthesis pathway (Kjer-Nielsen et al., 2012, Corbett et al., 2014). Specifically, 6-formyl pterin derived from photodegradation of folic acid and 5-(2-oxo-propylideneamino)-6-D-ribitylaminouracil (5-OP-RU) intermediate within the riboflavin biosynthesis pathway are the most well characterised MR1 binding antigens from both pathways (Corbett et al., 2014, Kjer-Nielsen et al., 2012). Critically, these antigens bind to and form a covalent Schiff bond within the MR1 antigenic cleft, resulting in the neutralisation of K43 residue and consequently permitting the translocation and trafficking of MR1 out of the ER (McWilliam et al., 2016b). The necessity of K43 neutralisation is further observed through another set of bacterial derived metabolites known as ribityllumazines which can occupy the MR1 ligand binding domain but fail to trigger ER egress as they fail to establish a Schiff bond (Awad et al., 2020, Mak et al., 2017, Keller et al., 2017a). This phenomena is also observed with the synthetic drug compound 3-([2,6-Dioxo-1,2,3,6- tetrahydropyrimidin-4-yl] formamido) propanoic acid (DB28), which also fails to create a Schiff bond; therefore retaining MR1 within the ER (Salio et al., 2020).

In a recent landmark study, host-derived bile acid metabolite with a microbiota-dependent sulfate modification: cholic acid-7 sulfate (CA7S) was discovered as the first endogenous MR1 ligand (Ito et al., 2024). Intriguingly, despite lacking the capacity for establishing a Schiff bond, CA7S was able to trigger MR1 surface expression, with

mutational modifications of the MR1 ligand binding domain revealed that both canonical MR1 ligand 5-OP-RU and CA7S occupied the same region of MR1 (Ito et al., 2024). It is important to note however that there was a several fold difference in concentration of CA7S required to stimulate MR1 surface expression compared to 5-OP-RU (Ito et al., 2024). Overall, the combination of Schiff bond requirement and ER stabilising proteins allows for a reservoir of ligand receptive immature MR1 that is capable of binding to increasingly diverse groups of organic as well as synthetic metabolite antigens.

1.4.2.2 MR1 egress from the ER

Following ligand binding, MR1 changes conformation and associates with β2m allowing for ER egress (McWilliam et al., 2016b). Ligand bound MR1 traffics through the Trans Golgi Network (TGN) and follows the default secretory pathway to reach the plasma membrane (McWilliam et al., 2016b, McWilliam and Villadangos, 2023). It has been proposed that some MR1 may also traffic through the endosomal pathway through the assistance of TGN-endosome associated proteins Rab6 and VAMP4 (Harriff et al., 2016). This endosomal pathway is proposed to play a role in how MR1 surveys and captures *Mycobacterium tuberculosis* (Mtb) derived ligands within endosomal compartments (Harriff et al., 2016).

1.4.2.3 The fate of MR1: Endocytosis, recycling and lysosomal destruction

The final stage of regulation for MR1 antigen presentation occurs at the plasma membrane. Independent of ligand affinity, 50% of surface MR1 complexes are endocytosed within 2-4 hours (Lim et al., 2022, McWilliam et al., 2016b). MR1 is internalised through the Clathrin-dependent endocytosis pathway mediated by plasma membrane resident adaptor protein 2 (AP2) (Lim et al., 2022). AP2 effectively binds

to conserved motifs within the cytoplasmic tail of plasma membrane proteins to actively initiate endocytosis. Indeed, proteins that utilise the AP2-clathrin endocytic such as CD1 molecules are endocytosed extremely rapidly (Barral and Brenner, 2007). The crucial detail however lies in the cytoplasmic tail of MR1 which possesses a hydrophobic tyrosine residue which makes for a suboptimal AP2 recognition motif (Lim et al., 2022). This suboptimal interaction between AP2 and cytoplasmic tail fundamentally regulates MR1 surface expression by slowing the rate of endocytosis enough to allow for antigen presentation but also actively terminating antigen presentation at a rate that mitigates unwarranted immune-activation (Lim et al., 2022, McWilliam and Villadangos, 2023).

Approximately 95% of endocytosed MR1 complexes are destroyed through the lysosomal degradation pathway (McWilliam et al., 2016b, Lim et al., 2022). The small portion of endocytosed MR1 that does not degrade is instead able to present new extracellular derived ligand (Karamooz et al., 2019). This pathway is dependent on Rab6 translocase through promotion of retrograde trafficking to the TGN (Huber et al., 2020).

Overall, MR1 antigen presentation is a tightly regulated system that is exquisitely dependent on ligand availability (McWilliam et al., 2016b). In this regard, MR1 is viewed as a “presentation on demand” system (McWilliam and Villadangos, 2023) that provides a real-time readout for perturbations in homeostatic cellular metabolic processes.

1.5 Sentinels of the mucosa: MAIT cells

The presentation of conserved antigenic signatures by MR1 precludes their presentation to conventional T cells, which by nature encode a virtually limitless diversity of TCRs. In 1993, Porcelli et al., discovered a population of T cells that expressed a semi-invariant TCR comprised of an α-chain variable region 7.2: $V_{\alpha}7.2$ (TRAV1-2), coupled to an α-chain joining region 33: $J_{\alpha}33$ (TRAJ33) (Porcelli et al., 1993). They hypothesised this population to: "*recognize a limited spectrum of antigens and suggests that they may use nonpolymorphic antigen-presenting molecules"* (Porcelli et al., 1993). A pivotal study by Treiner et al., later identified the enrichment of this semi-invariant T cell population to the gut lamina propria of humans and mice; therefore coining this population as Mucosal-associated Invariant T (MAIT) cells (Treiner et al., 2003). Importantly, MAIT cells were demonstrated to be MR1 restricted, with a profound absence of MAIT cells in germ-free mice suggesting an ontogenic dependence on MR1 dependent presentation of microbiota derived antigens (Treiner et al., 2003). In the years since, work has identified MAIT cells as the most abundant unconventional T cell population that is broadly enriched across all mucosal barrier locations throughout the host (Dusseaux et al., 2011, Terpstra et al., 2020, Gibbs et al., 2017, Booth et al., 2015, Jabeen et al., 2022). Thus, through barrier localisation, detection of broadly conserved riboflavin metabolites presented by MR1 and innate sensitivity to micro-environmental pro-inflammatory cues, MAIT cells are uniquely positioned to playing a critical role in pathogen surveillance and eradication.

1.5.1 MAIT cell development and localisation

Consistent with conventional T cells, MAIT cells are selected within the thymus. (Martin et al., 2009). However, unlike conventional T cells, MAIT cells follow a distinct development and selection pathway that is deeply influenced by an integration of environmental cues from the host microbiome (Legoux et al., 2020). This is evident through the near absence of MAIT cells detected in germ free mice (Treiner et al., 2003). MAIT cells are selected by MR1 expressing $CD4^+/CD8^+$ (double positive) thymocytes (Seach et al., 2013, Martin et al., 2009). Legoux et al, illuminated that 5-OP-RU can be rapidly trafficked from distal mucosal and skin sites to the thymus and be presented by double positive thymocytes in an MR1 dependent manner (Legoux et al., 2019). Critically, the ability for riboflavin antigens to travel across distinct tissue sites raises the intriguing possibility that microbial translocation across one mucosal interface may activate or prime anatomically distant MAIT cells in essentially an endocrine manner. This may play a pivotal role for MAIT cells during infection with organisms such as viruses which do not synthesise riboflavin, as further examined in Sections 1.7 and 1.8. Furthermore, mono-colonisation of germ free mice with *E.coli* lacking the riboflavin operon failed to induce MAIT cell selection, therefore highlighting a fundamentally governing role of riboflavin exposure in MAIT cell selection (Legoux et al., 2019). Interestingly, microbial colonisation of adult germ free mice restored thymic MAIT cell development; however these MAIT cells failed to expand and populate mucosal sites such as the skin and lung (Legoux et al., 2019).

Following thymic selection, MAIT cells display a tissue homing program acquired through the expression of master transcription factor promyelocytic leukemia zinc finger protein (PLZF) (Koay et al., 2016), as well expression of tissue targetting

chemokine receptors CCR6, CCR5 and CXCR6 (Salou et al., 2019, Lantz and Legoux, 2019). Furthermore, MAIT cells also strongly express the transcription factor bZIP (C/EBPδ), allowing for highly efficient extravasation from circulation into inflamed tissue sites (Lee et al., 2018). Following thymic egress, MAIT cells rapidly continue to expand, differentiate and populate mucosal sites until approximately thirty years of age, before a steady age associated decline in frequency (Gherardin et al., 2018, Ben Youssef et al., 2018, Novak et al., 2014). Within humans, MAIT cells make up to 2-4% in lung, 20-50% in liver, up to 2% in skin, and up to 60% of $CD4^-$ T cells in jejunum (Dusseaux et al., 2011, Kurioka et al., 2016, Provine and Klenerman, 2020). Thus, early riboflavin synthesising microbial colonisation of the host commands MAIT cell thymic development and subsequent expansion to barrier sites (Legoux et al., 2020, Legoux et al., 2019, Constantinides et al., 2019).

1.5.2 Primed for response: The resting MAIT cell phenotype

Whilst murine MAIT cells are lineage committed within the thymus to function as either T helper (Th) type 1 or Th17 cells (Rahimpour et al., 2015, Cui et al., 2015, Chandra et al., 2023, Riffelmacher et al., 2023), human MAIT cells do not follow functional bifurcation and are predominantly homogenous with only modest variances in phenotype reported (Dias et al., 2017). MAIT cells uniformly express of $CD161^{++}$, IL-18 receptor, CD26 and TCR alpha chain Vα7.2 (Dusseaux et al., 2011, Martin et al., 2009). In human blood, most MAIT cells are $CD8^+$ or double negative ($CD8^-/CD4^-$), with a very small minority expressing either $CD4^+$ or double positive ($CD8^+/CD4^+$) (Gherardin et al., 2018). Furthermore, the $CD8^+$ MAIT cell population is enriched with CD8$\alpha\alpha$ homodimer (Walker et al., 2012), which has recently been revealed to interact with MR1 and enhance antigen responsiveness (Souter et al., 2022). This finding

correlates with the observation that $CD8^+$ MAIT cells display greater functional response to riboflavin synthesising bacteria compared to double negative MAIT cells (Dias et al., 2018). Furthermore, circulating MAIT cells also display a bimodal expression of markers such as CD56, CD84 and CD94, with the positive expression of these markers associated with greater response to cytokine stimulation (Dias et al., 2017). Unlike conventional T cells, the co-expression of several usually mutually exclusive transcription factors by resting MAIT cells underpins a uniquely polyfunctional response potential that can be enacted upon several modalities of stimulation (Figure 5).

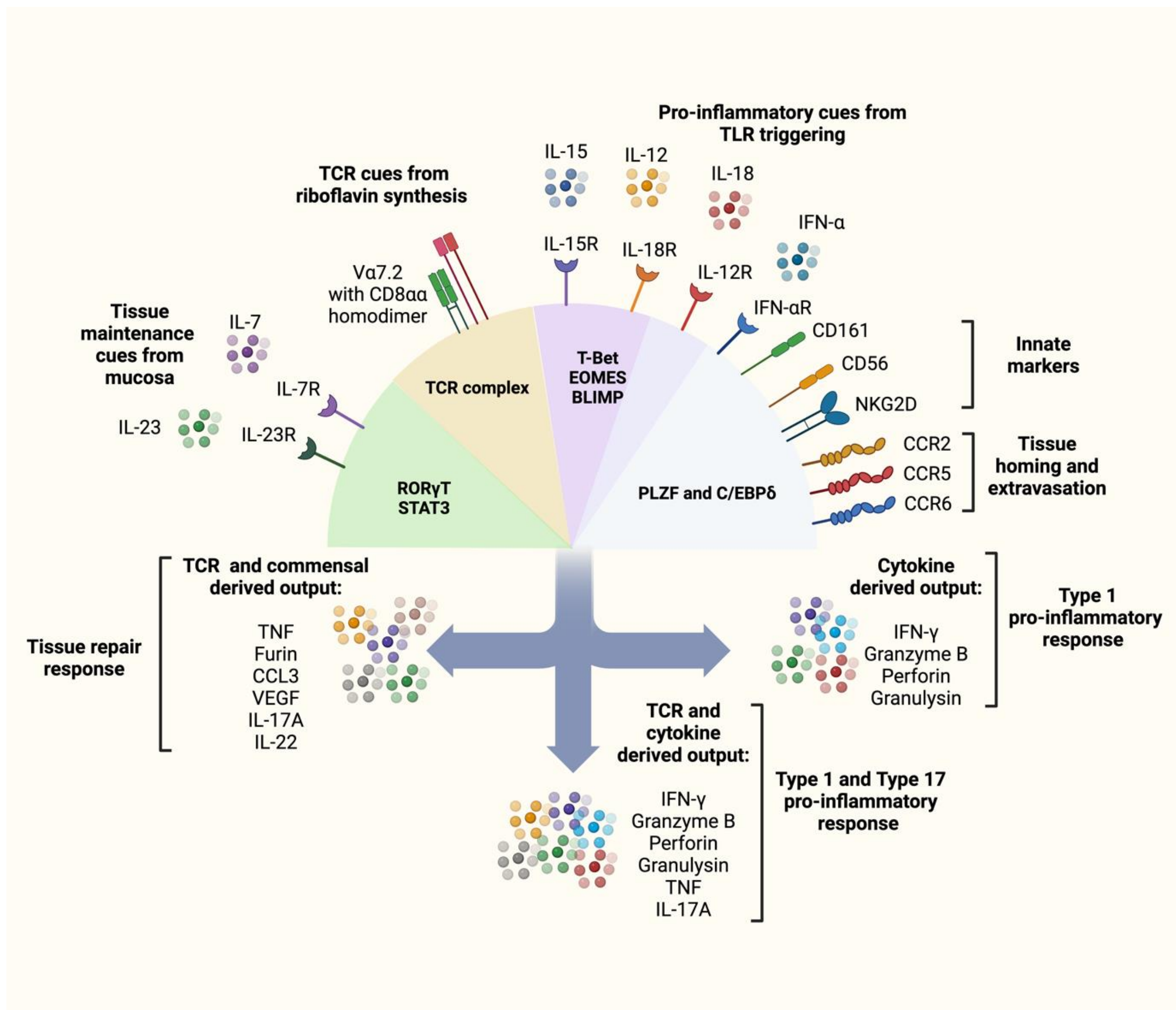

Figure 5. MAIT cells are primed for polyfunctional responses

MAIT cells co-express several transcription factors that enable response to several modalities of stimulation. Expression of PLZF and C/EBPδ allows for an extravasation and tissue homing profile as well as expression of several innate markers and cytokine receptors. T-bet, EOMES and BLIMP program allows for expression of several type 1 cytokine receptors. MAIT cells also express a semi invariant T cell receptor (TCR) most commonly associated with the co-receptor CD8 homodimer. This complex allows for MAIT cells to respond to metabolite antigens derived from the riboflavin synthesis pathway. MAIT cells also concurrently express RORγt and STAT3 transcription factors allowing for detection of type 17 cytokines. Type 1 cytokine stimulation of MAIT cells drives a T-bet/EOMES mediated response characterised by IFN-γ and granzyme B. Stimulation of MAIT cell TCR either in isolation or in combination with type 17 cytokines drives a tissue repair response. Whilst combined stimulation of MAIT TCR and type 1 cytokines drives a sustained and maximal response of several type 1 and 17 mediators as well as cytolytic capacity. *Figure created using Biorender.*

Human MAIT cells co-express the following transcription factors: PLZF, Eomesodermin (EOMES), B-lymphocyte induced maturation protein-1 (BLIMP-1), Signal transducer and activator of transcription 3 (STAT3), T-box transcription factor TBX21 (T-bet), RAR-related orphan receptor gamma (RORγt) and CCAAT/enhancer binding protein-deta (C/EBPδ) (Billerbeck et al., 2010, Leeansyah et al., 2015a, Lamichhane et al., 2019, Lee et al., 2018). The co-expression of T-bet, EOMES and BLIMP-1 allows for a coordinated production of type 1 immune response mediators such as granzyme B, perforin (both cytotoxic molecules) as well as cytokines like IFN-γ (Szabo et al., 2000, Pearce et al., 2003, Kallies et al., 2009, Rutishauser et al., 2009). This is further reflected in high expression of type 1 cytokine IL-12 receptor (IL-12Rβ1) and IL-18 receptor (Fergusson et al., 2014). Overall, this allows MAIT cells to effectively function in a manner reminiscent of type 1 cytotoxic $CD8^+$ T cells.

Curiously, the simultaneous expression of type 17 master regulators RORγT (Ivanov et al., 2006) and STAT3 (Wilson et al., 2015) allows for MAIT cells to function as Th17 T cells. This is reflected through the production of type-17 cytokines such as IL-17 and IL-22 upon activation (Billerbeck et al., 2010). It is important to note that IL-17 and IL-22 expression by circulating MAIT cells is more tightly regulated (Dusseaux et al., 2011) and requires a combination of TCR stimulation with whole bacteria along with accessory signals such as IL-1β or IL-7 (Leeansyah et al., 2015a, Gibbs et al., 2017, Turtle et al., 2011, Gracey et al., 2016, Tang et al., 2013). Furthermore, type 17 functionality by MAIT cells is likely dependent on localised cues derived from anatomically distinct sites demonstrated by mouth and female genital tract originating MAIT cells which are type 17 biased upon activation (Gibbs et al., 2017, Sobkowiak et al., 2019). Additionally, MAIT cells express IL-23R (Billerbeck et al., 2010); a receptor

for the canonical type 17 cytokine IL-23 (Korn et al., 2009). Finally, the expression of PLZF provides MAIT cells with the ability to be activated by cytokines through elevated baseline expression of cytokine receptors such as IL-18Rα and IL-12β (Mao et al., 2016).

The linkage of PLZF and cytokine receptor expression is conserved in other innate and innate-like lymphocyte populations such as gamma delta T cells ($\gamma\delta$ T cells), invariant Natural Killer T cells (iNKT cells) and NK cells (Gutierrez-Arcelus et al., 2019, Kovalovsky et al., 2008, Provine et al., 2018). Thus, resting MAIT cells remain flexibly mobilised for a polyfunctional response through a coordinated expression network of several transcription factors.

1.5.3 MAIT cell activation

Borrowing fundamental characteristics from both innate and adaptive lymphocytes; MAIT cells can be activated through both TCR-dependent as well as TCR-independent pathways. Importantly, MAIT cells are able to leverage and integrate signals from both distinct modes of stimuli to generate a sustained polyfunctional response (Figure 6).

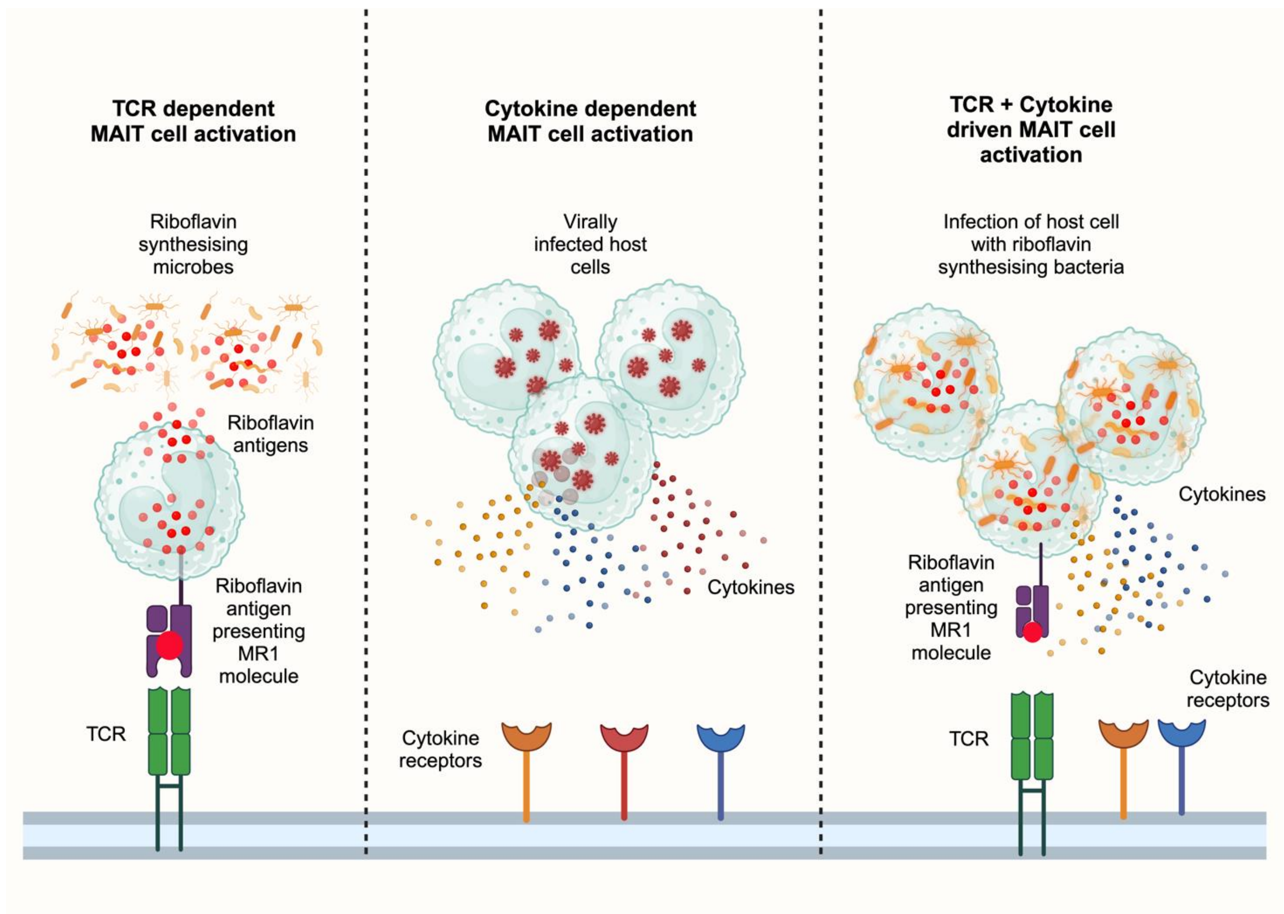

Figure 6. MAIT cell activation modalities
MAIT cells can be activated through several pathways. TCR dependent activation of MAIT cells occurs through MR1 mediated presentation of riboflavin antigens derived from riboflavin synthesising bacteria. Cytokine dependent activation of MAIT cells can occur during viral infections as virally infected cells express pro-inflammatory cytokines which can be captured by cytokine receptors expressed on MAIT cells. Finally, MAIT cells can be activated through a combination of TCR and cytokine signals, as observed with intracellular infection of host cells with riboflavin synthesising microbes which promotes intracellular MR1 capturing and presenting of riboflavin ligands, as well as driving secretion of cytokine production by infected cell.
Figure created using Biorender.

1.5.3.1 TCR dependent activation of MAIT cells

Like all T cells, MAIT cells can be activated through TCR ligation with their cognate antigen presenting molecule; in this case MR1 (Corbett et al., 2014, Kjer-Nielsen et al., 2012). Unlike conventional T cells, all MAIT cells within the periphery are memory programmed (Dusseaux et al., 2011) and therefore do not require priming events such as antigen presentation in secondary lymphoid organs (Wang et al., 2018a). To counterbalance this, MAIT cell TCR signalling is tightly regulated, and even sustained signalling fails to induce prolonged cytokine release or proliferation (Turtle et al., 2011). TCR stimulation rapidly activates MAIT cells within a few hours as per CD69 expression, and generates a rapid inflammatory response characterised by IFN-γ, TNF, and granulocyte-macrophage colony-stimulating factor (GM-CSF) production that peaks at 6 hours before a steady decline (Lamichhane et al., 2019). This phenotype is further reflected through increased expression of transcription factors: T-bet and RORγT (Lamichhane et al., 2019). Furthermore, through degranulation (CD107a), granzyme B and perforin expression; MAIT cells can enact direct cytolytic killing through TCR engagement (Le Bourhis et al., 2013, Kurioka et al., 2015). In the absence of additional pro-inflammatory signals, TCR stimulation of MAIT cells is believed to drive a sustained tissue repair homeostatic response (Hinks et al., 2019, Leng et al., 2019, Constantinides et al., 2019): as reviewed in Section 1.6. Overall, TCR stimulation drives a rapid but short-lived release of several cytokines and cytolytic engagement that resolves into a tissue repair signature.

1.5.3.2 TCR independent activation of MAIT cells

As reviewed in Section 1.5.2, the high expression of several interleukin receptors such as IL-18R, IL-12R, IL-7R, IL-15R, IL-23R and IFN-αR (Dias et al., 2017, Leeansyah et al., 2015a, Dias et al., 2018, Gherardin et al., 2018) likely predisposes MAIT cells with an innate sensitivity for response to local and systemic cytokine release during infection. In congruence, a pivotal study by Ussher et al., demonstrated that MAIT cells can be activated by cytokine stimulation in a TCR-independent manner (Ussher et al., 2014). Importantly, more than two cytokines are required for the activation of MAIT cells, with the combination of IL-12/IL-18 to be the first and most well described (Ussher et al., 2014). Subsequent studies have shown IL-15, IFN-α/β, TNF as well as the gut associated cytokine TNF-like protein 1 (TL1A), to drive a MAIT cell response in combination with IL-18 and/or IL-12 (Leng et al., 2019, Slichter et al., 2016, Sattler et al., 2015, Van Wilgenburg et al., 2016, Provine et al., 2021, Lamichhane et al., 2020). In contrast to TCR driven activation, the MAIT cell response to cytokine stimulation is slower and restricted to IFN-γ, granzyme B and perforin expression (Lamichhane et al., 2019). Critically, cytokine driven activation of MAIT cells fundamentally allows for a functional response to non-riboflavin synthesising pathogens such as viruses; as further explored in Section 1.7.1.

1.5.3.3 Synergistic activation of MAIT cells

Barring viral infections, most microbial infections are accompanied with riboflavin synthesis as well as cytokine production from infected host cells (Konecny et al., 2024, Pavlovic et al., 2020, López-Rodríguez et al., 2023) Unsurprisingly, MAIT cells leverage combined TCR, cytokine and TLR stimulation to generate a robust and dynamic response (Provine and Klenerman, 2020, López-Rodríguez et al., 2023).

Several murine *in vivo* studies have demonstrated that inoculation with synthetic 5-OP-RU along with co-delivery of inflammatory stimuli such as TLR agonists, activates, expands and drives pulmonary MAIT cell accumulation (Chen et al., 2017, Nelson et al., 2023, Hinks et al., 2019). Furthermore, addition of cytokines such as IL-7, IL-12 and IL-18 during TCR stimulation potentiates MR1 driven bacterial activation and cytotoxic response by MAIT cells (Leeansyah et al., 2015a, Wallington et al., 2018, Kurioka et al., 2018, Jo et al., 2014, Jesteadt et al., 2018).

Synergistic MAIT cell stimulation drives the expression of a diverse breadth of cytokines IFN-γ, TNF, IL-17, GM-SCF (Le Bourhis et al., 2010, Chua et al., 2012, Dusseaux et al., 2011), enhances cytotoxic capacity (Dusseaux et al., 2011, Kurioka et al., 2015), and increases release of chemokines such as CXCL1, CCL3, CCL4 and CXCL16 (Slichter et al., 2016, Hinks et al., 2019). It is important to note that MAIT TCR ligands are abundantly available within the host due to riboflavin synthesis by resident microbiota, therefore the integration of additional inflammatory cues allows for a finely tuned MAIT cell response.

1.6 MAIT-TCR driven antimicrobial and homeostatic responses

Several murine *invivo* studies have shown MAIT cell mediated clearance of intracellular bacterial pathogens such as *Mycobacterium abscessus* (Le Bourhis et al., 2010)*, Mycobacterium bovis* bacillus Calmette-Guerin (BCG) (Chua et al., 2012), *Francisella tularenis (F. tularenis)* (Meierovics et al., 2013, Zhao et al., 2021, Meierovics and Cowley, 2016)*, Legionella longbeache* (Wang et al., 2018b)*, Klebsiella pneumonia* (Georgel et al., 2011), and *Escherichia coli* (*E.coli*) associated urinary tract infection (Cui et al., 2015). Within humans, bacterial infections are associated with

decreased frequencies of circulating MAIT cells and concomitant recruitment at tissue sites of infection (Grimaldi et al., 2014). Furthermore, separate studies on human cohorts challenged with wild type *Salmonella enterica* serovar Typhi (*S. typhimurium*) have shown increased activation, proliferation and more pronounced tissue homing characteristics in circulating MAIT cells (Howson et al., 2018, Salerno-Goncalves et al., 2017). Intriguingly, clonal expansion of distinct MAIT TCRβ clonotypes emerged during peak *S. typhimurium* infection suggesting pathogen specific adaptations and response (Howson et al., 2018). Indeed, this was initially shown by Gold et al., whereby selective expansion of MAIT clonotypes within the TCRβ chain was specific to the distinct classes of microbes used within the study: *Mycobacterium smegmatis, Candida albicans and S. typhimurium* (Gold et al., 2014). Furthermore, assessment of a cohort in Vietnam carrying a polymorphic mutation in the MR1 gene, demonstrated increased susceptibility to *M. tuberculosis* infection, therefore implicating MAIT cells in playing a critical role in the control of this disease (Seshadri et al., 2017).

Beyond direct inflammatory and cytolytic control, MAIT cells can also perform several helper and promoter roles that allow for indirect control of infection such as: neutrophil recruitment (Lamichhane et al., 2019), neutrophil differentiation into APCs (Davey et al., 2014), promoting tissue migration and differentiation of monocytes into DCs (Meierovics and Cowley, 2016). The latter response induced $CD4^+$ and $CD8^+$ T cell pulmonary accumulation and clearance of murine *F. tulaneris* infection (Meierovics et al., 2013, Meierovics and Cowley, 2016).

Whilst MAIT cells are able to enact a powerful pro-inflammatory response to several riboflavin synthesising pathogens, a contrasting response is evoked when interacting

with commensal microbiota. Instead, colonisation of germ-free mice with skin commensal *Staphylococcus epidermis* drove expansion and cutaneous accumulation of MAIT cells with a tissue repair signature (Constantinides et al., 2019). This signature was functionally validated through a murine *invivo* wound model whereby MAIT cells treated with topically applied 5-OP-RU displayed enhanced closure of punch- biopsy cutaneous wounds (Constantinides et al., 2019). In congruence with these findings, transcriptomic analysis by Hinks et al., revealed that both human and murine TCR stimulated MAIT cells display a conserved tissue repair signature (Hinks et al., 2019). Furthermore, supernatants from E.coli stimulated MAIT cells accelerated wound closure of an intestinal epithelial cell line monolayer (Leng et al., 2019).

Remarkably, this dual functionality of inflammation and tissue repair by MAIT cells is shared across humans, mice, rats, sheep, cattle, and opossums, therefore indicating a polyfunctional response to riboflavin synthesis that is evolutionarily conserved across 110 million years of evolution (Bugaut et al., 2023).

1.7 MAIT cells in viral diseases

A range of *in vitro* viral infection studies have now demonstrated cytokine dependent MAIT cell activation and ensuing pro-inflammatory response, thus suggesting MAIT cells play a powerful anti-viral immune-protective role. However, there is a disparity of MAIT cell responses observed when comparing patient cohorts suffering from acute compared to chronic viral infections (Figure 7), suggesting a far more complex relationship between MAIT cell function and virus; one that is temporally based.

1.7.1 MAIT cells during *in vitro* and acute viral infection settings

During acute HIV infection, MAIT cells are highly activated, and expanded with enhanced function (Lal et al., 2020). Whilst *in vitro*, MAIT cells are able to mount an anti-viral response against HIV through chemokine production of CCL3, CCL4 and CCL5 in a cytokine dependent manner (Phetsouphanh et al., 2021). Furthermore, Hepatitis C virus (HCV) infected APCs activate MAIT cells through an IL-18 and IL-15 dependent manner *in vitro*, resulting in IFN-γ production and subsequent suppression of HCV replication (Van Wilgenburg et al., 2016). The authors also examined MAIT cells from HCV infected patients undergoing IFN-α treatment and reported increased activation (as per CD69 expression) compared to untreated HCV patients; therefore also implicating a role for IFN-α to potentiate MAIT cell response (Van Wilgenburg et al., 2016). The same study also found peripheral blood MAIT cells isolated from patients with acute dengue virus (DENV) and Influenza A (IAV) infection exhibiting an activated profile marked by granzyme B expression (Van Wilgenburg et al., 2016). The response of MAIT cells during Influenza was further demonstrated in a separate study of patients hospitalized with IAV, which found higher frequencies of circulating MAIT cells to be associated with greater recovery from infection (Loh et al., 2016). This response was further verified *in vivo*, whereby MAIT cells conferred protection to mice against a lethal dose of Influenza (van Wilgenburg et al., 2018). Within this study, MAIT cells accumulated within the lungs and demonstrated heightened CD69, CD25 and granzyme B expression; this phenotype was primarily driven by IL-12 (van Wilgenburg et al., 2018).

Comparatively, another respiratory virus, Measles virus, directly infects and programs MAIT cells for apoptosis *in vitro* (Rudak et al., 2021). This was the first report that

demonstrated viral infection of MAIT cells, therefore raising the intruiging possibility that other lymphotropic viruses may also have the capacity to infect MAIT cells. In Chapter 4 we investigate the susceptibility of MAIT cells to VZV infection.

Surprisingly, MAIT cell killing of of Hepatitis B virus (HBV) antigen-expressing hepatocytes *in vitro* was demonstrated to be MR1-dependent (Liu et al., 2020). This was concurrent with increased MR1 transcript and MR1 protein being detected in HBV infected liver tissue; therefore suggesting a direct role for the MR1-MAIT cell axis (Liu et al., 2020). In contrast, hepatic MAIT cells isolated from patients with acute Hepatitis A show an activated and cytotoxic phenotype (Rha et al., 2020). This response was IL-15 dependent and mediated through upregulation of NKG2D receptors on MAIT cells allowing for TCR-independent killing against cells displaying stress or damage (Rha et al., 2020). The authors further proposed this IL-15 - MAIT NKG2D axis to be a major contributor to liver injury during viral hepatitis (Rha et al., 2020).

More recently, MAIT cells were revealed to upregulate granzyme B and the degranulation marker CD107a towards SARS-COV2 infected monocyte derived macrophages in a partially MR1-dependent manner (Flament et al., 2021). The authors speculated this MR1-dependent MAIT cell response to be driven through an altered host metabolome rather than a direct viral ligand presented by MR1. This theory remains consistent with a recent report that demonstrates no activation of MAIT cells when exposed to purified SARS-COV2 virions (Huang et al., 2024). Cumulatively, MAIT cells are activated and enact an anti-viral response during a range of viral infections within an acute setting.

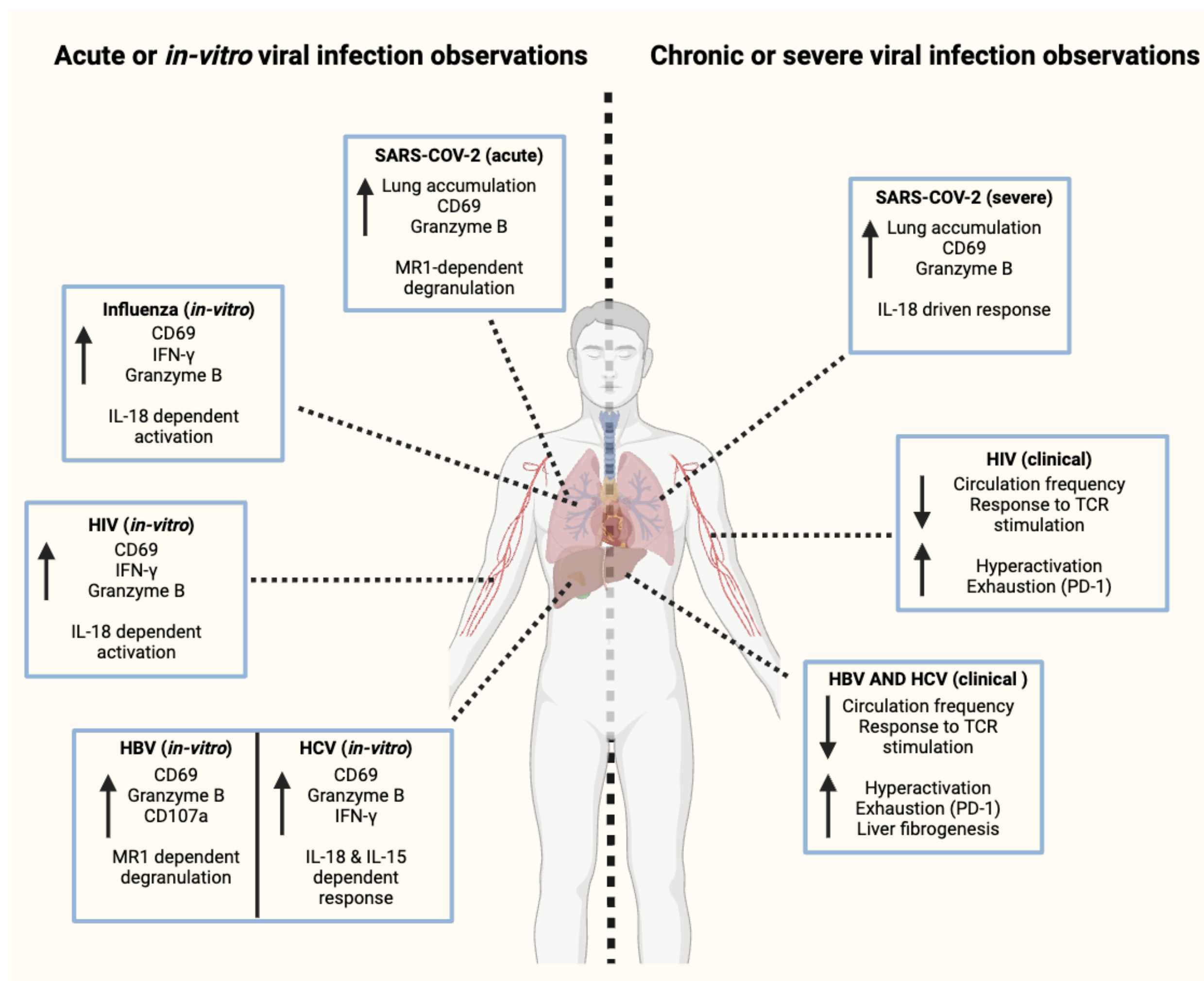

Figure 7. MAIT cells in viral diseases
Observations from acute or *in vitro* studies of MAIT cells during viral infections such as HIV, Influenza and HCV demonstrate cytokine dependent activation accompanied with an anti-viral response denoted by IFN-γ and granzyme B expression. HBV and SARS-COV2 infections also drive a TCR-dependent MAIT cell activation *in vitro* that results in cytolytic killing of target cell. Observations of MAIT cells during chronic viral infections (HIV, HBV, HCV and SARS-COV2) consistently reveal marked circulatory reduction, hyper activated and exhausted profile accompanied with functionally dysregulated response to TCR stimulation.
Figure created using Biorender.

1.7.2 MAIT cells in chronic viral settings

A common theme observed across all chronic viral infections studied thus far is the profound loss of circulating MAIT cells accompanied with a pronounced hyperactivated, exhausted and dysfunctional profile. Whether the reduction of

circulating MAIT cells is a product of death or recruitment to barrier locations is not completely understood and is likely specific to the type of viral infection.

Several studies have demonstrated a consistent decrease of circulating MAIT cells during chronic HIV infection (Leeansyah et al., 2013, Cosgrove et al., 2013, Lal et al., 2020, Spaan et al., 2016). Furthermore, this decline is persistent as longitutidinal patient cohort studies have observed limited recovery of circulating MAIT cell numbers is observed in patients over time as they undergo anti-retroviral therapy (ART) (Spaan et al., 2016, Phetsouphanh et al., 2021). The reduction of circulating MAIT cells maybe explained by a recent finding which revealed that MAIT cells undergo activation induced pyroptosis in response to HIV virions (Xia et al., 2022). Additionally, the small subset of $CD4^+$ cells within the overall MAIT cell pool are susceptible to HIV infection and can contribute to the latency reservoir (Wu et al., 2023).

Interestingly, whilst MAIT cells in a chronic HIV setting maintain a functional capacity towards cytokine stimulation, their response to TCR driven activation is greatly diminished (Lal et al., 2020). A hallmark of chronic HIV infection is sustained IFN-α expression (Lehmann et al., 2010) which in turn induces a counter-balancing IL-10 driven immune-suppressive response by monocytes (Granelli-Piperno et al., 2004). Critically, this chronic exposure to IL-10 production impairs TCR driven CD107a and granzyme expression by MAIT cells in response to *E.coli* stimulation (Tang et al., 2020). It is speculated that this impaired response by MAIT cells to riboflavin synthesis during chronic HIV may compromise the host to secondary bacterial infections (Samer et al., 2021, Tang et al., 2020).

In the context of chronic HCV infection, or HCV/HIV co-infection, circulating MAIT cells are severely depleted and exhibit a highly activated, but diminished IFN-γ phenotype which does not recover even after ART treatment (Spaan et al., 2016, Hengst et al., 2016, Barathan et al., 2016, Cannizzo et al., 2019). Furthermore, another study demonstrated a depletion of intra-hepatic MAIT cells in HCV infected patients, therefore also indicating a localised loss of MAIT cells (Bolte et al., 2017). Interestingly, the loss of MAIT cells was inversely correlated with liver fibrosis and inflammation, with the remaining MAIT cells displaying a highly activated profile (Bolte et al., 2017). Similarly, chronic HBV infection is also associated with decreased circulating MAIT cells, with the remaining MAIT cells exhibiting an exhausted phenotype (Huang et al., 2020, Yong et al., 2018). Furthermore, the loss of peripheral MAIT cells is further exacerbated during a HBV/HDV co-infection (Dias et al., 2019).

A consequence of chronic HBV and HCV infection is the impaired MAIT cell response to TCR-dependent stimulation (Bolte et al., 2017, Hengst et al., 2016, Liu et al., 2020). In the case of HBV, the sustained release of conjugated bilirubin derived from liver damage promotes MAIT cell activation and apoptosis whilst inhibiting TCR-dependent proliferation (Liu et al., 2020). It is likely the release of conjugated bilirubin during HCV infection also interferes with MAIT TCR responses in a similar manner.

Recently, a striking relationship between MAIT cells and severe COVID infection has emerged. Several studies of patients with severe COVID infection have revealed a substantial loss of circulating MAIT cells, accompanied with a concurrent pulmonary enrichment of activated and cytotoxic MAIT cells (Flament et al., 2021, Parrot et al., 2020, Jouan et al., 2020). This recruitment of MAIT cells to the infection site however

seems to have a deleterious outcome, as the activation of MAIT cells has been correlated with poor or even fatal clinical outcomes (Parrot et al., 2020, Youngs et al., 2021, Flament et al., 2021). Flament et al, suggested this outcome to likely be driven by heightened IL-18 dependent MAIT cell cytotoxicity (Flament et al., 2021). Post COVID infection, there is an impaired level of MAIT cell frequency recovery with a persistent functional impairment to cytokine stimulation observed, therefore potentially compromising host MAIT cell driven responses to other infections (Kammann et al., 2023).

However, in a separate study a milder COVID infection outcome in females compared to males was correlated to a greater quantitative and qualitative MAIT cell response by females (Yu et al., 2021). This raises the possibility that MAIT cells may play a sexually dimorphic nuanced role in viral infections. Furthermore, MAIT cell numbers and functional capacity are positively correlated with magnitude of $CD4^{+}$ T cell and antibody response to SARS-COV2 vaccination, suggesting that MAIT cells are associated with the induction and quality of response to vaccination (Boulouis et al., 2022). Indeed, this concept of MAIT cell adjuvant activity is seen with an adenovirus based vaccine system where increased activation of MAIT cells was positively correlated with vaccine mediated T cell responses in humans (Provine et al., 2021). Interestingly, our group revealed that detection of MAIT cell frequencies in patients with HCMV reactivation following haematapoetic stem cell transplant (HSCT) could accurately discriminate between high level HCMV reactivation versus low level HCMV reactivation (Stern et al., 2022). Therefore, raising the intriguiging possibility that MAIT cell frequencies can potentially serve as surrogate biomarkers of viral disease outcome.

In summation, it appears that MAIT cells are activated and play a protective role during acute infection, but exert a potentially immune-pathological dysfunctional role in persistent and chronic settings across several viral diseases.

1.8 Master manipulators: Herpesvirus interactions with the MR1-MAIT cell axis

A priori assumptions of the MR1 ligand repertoire suggests a limited role of the MR1-MAIT TCR axis in host anti-viral immunity: *"MAIT cells are activated, in an MR1-dependent manner, by a broad spectrum of bacteria and yeast, but not by viruses"* (Kjer-Nielsen et al., 2012). Whilst this remains true in a biologically isolated *in vitro* setting, several *in vivo* viral infections such as VZV, HSV-1, HIV, HCV, DENV and CMV can compromise the structural integrity of mucosal interfaces therefore increasing microbial translocation (Brenchley et al., 2006, Townsend et al., 2021, Sandler et al., 2011, van de Weg et al., 2013, Ramendra et al., 2020, Diniz et al., 2018, Möckel et al., 2022). The direct consequence of barrier breakdown is evident during acute HIV infection whereby MAIT cell activation and expansion in circulation and mucosa directly correlated with microbial translocation (Lal et al., 2020). Ultimately, this sets a selective evolutionary pressure for viruses to actively target MR1 antigen presentation to consequently mitigate TCR-dependent MAIT cell responses (Samer et al., 2021).

As reviewed in Section 1.4.2.1, immature MR1 awaits ligand binding within the ER. In a landmark study by McSharry et al, our lab demonstrated that several herpesviruses such as HSV-1 and CMV are able to effectively target this ER reservoir of immature MR1 (McSharry et al., 2020). This depletion of the ligand receptive MR1 pool

profoundly compromised presentation of MR1 ligands. Functionally, HSV-1 infection and modulation of MR1 antigen presentation extended to an impaired MAIT TCR recognition of *E.coli* treated cells. The targetting of MR1 by HSV-1 appears to be ER based, as surface ligand bound MR1 remained impervious throughout infection. Surprisingly, CMV effectively targetted both immature and mature ligand bound forms of MR1. HSV-1 protein US3 was demonstrated to partially mediate MR1 downregulation (McSharry et al., 2020). In a separate study, our lab also revealed CMV glycoprotein gpUS9 plays a partial role in downregulating total MR1 expression, whilst also further demonstrating that CMV derived MR1 modulation disrupts TCR-dependent MAIT cell activation (Ashley et al., 2023). More recently, our group established that HSV-1 encoded virion host shutoff (vhs) protein degrades MR1 transcripts, while ICP22 consigns immature MR1 to proteosomal degradation (Samer et al., 2024b). The loss of MR1 transcripts and immature protein was also mirrored during infection with the closely related HSV-2 virus (Samer et al., 2024b). Thus, several herpesviruses have evolved effective strategies to MR1 expression. With this knowledge, we therefore assessed the capacity for VZV to modulate MR1 antigen presentation in Chapter 2 of this thesis.

1.9 Concluding remarks, hypothesis and project aims

MAIT cells are enriched at key anatomical sites of varicella and herpes zoster infection, and are armed to abundantly secrete a diverse suite of pro-inflammatory mediators that pose a threat to effective viral replication. However, through co-speciation spanning several millennia, VZV has evolved several diverse strategies to become a master manipulator of host detection and effector systems. At the time of commencing this project, our understanding into direct viral impacts upon the MR1-MAIT cell axis was germinal and limited to an initial report describing MR1 modulation by HSV-1 and HCMV. Given the previous reports of VZV disrupting classical MHC presentation as well as close evolutionary relationship to other herpesviruses, we chose to examine whether VZV also targets MR1 antigen presentation. Furthermore, we also explored the possibility of VZV infecting MAIT cells themselves and the functional consequences deriving from any such interaction.

Thus, the aims of this doctoral thesis are:

1. To examine MR1 antigen presentation during VZV infection (Chapter 2).

2.To expand our understanding on the repertoire of VZV lymphotropism by investigating MAIT cell infection (Chapter 3).

3. To evaluate the functional consequences of VZV interaction with MAIT cells (Chapter 4).

The aims of this study attempt to fulfil the hypothesis that VZV modulates the metabolite antigen sensory system (MR1) as well as productively infects and suppresses MAIT cell functional response; therefore profoundly negating the host MR1-MAIT cell axis.

Chapter 2. Suppression of MR1 antigen presentation by Varicella Zoster Virus

2.1 Introductory statement

The ability for VZV to counter classical anti-viral responses of T cells and NK cells through subverting their cognate recognition systems has been previously established (as reviewed in Chapter 1.3.1). However, there is a dearth of studies that examine how VZV interacts with non-classical antigen presenting systems such as MR1 which presents riboflavin synthesis metabolite ligands. Whilst viruses do not synthesise riboflavin, our lab previously demonstrated in a landmark study that two human herpesviruses: HSV-1 and HCMV, can profoundly suppress MR1 expression. Building upon this work, we investigated the capacity for VZV to also modulate MR1 antigen presentation. Following an initial examination during my Honours year of how VZV interacts with the host MR1 antigen presentation pathway, we extended these observations during the first year of my PhD. The findings generated from both my Honours and first year of PhD culminated into a publication in the Journal of Infectious Diseases, and forms the first chapter of my thesis results. The materials and methods used for generating the data within this publication are all enclosed as a subsection within the paper and not a separate thesis chapter. Similarly, all literature citations and figures associated with supplementary information are included as subsections within the paper.

The findings of Chapter 2 not only address the question of whether VZV modulates MR1, but also bolsters a new avenue of examining viral regulation of non-peptide antigen presenting pathways.

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Varicella Zoster Virus Impairs Expression of the Nonclassical Major Histocompatibility Complex Class I–Related Gene Protein (MR1)

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The antigen presentation molecule MR1 (major histocompatibility complex, class I–related) presents ligands derived from the riboflavin (vitamin B) synthesis pathway, which is not present in mammalian species or viruses, to mucosal-associated invariant T (MAIT) cells. In this study, we demonstrate that varicella zoster virus (VZV) profoundly suppresses MR1 expression. We show that VZV targets the intracellular reservoir of immature MR1 for degradation, while preexisting, ligand-bound cell surface MR1 is protected from such targeting, thereby highlighting an intricate temporal relationship between infection and ligand availability. We also identify VZV open reading frame (ORF) 66 as functioning to suppress MR1 expression when this viral protein is expressed during transient transfection, but this is not apparent during infection with a VZV mutant virus lacking ORF66 expression. This indicates that VZV is likely to encode multiple viral genes that target MR1. Overall, we identify an immunomodulatory function of VZV whereby infection suppresses the MR1 biosynthesis pathway.

Keywords. varicella zoster virus; VZV; immune modulation; MR1.

Innate-like T cells often express a semi-invariant T-cell receptor (TCR) and are restricted by monomorphic major histocompatibility complex, class I (MHC-I)–like molecules, allowing for rapid responses to conserved antigens [1]. MHC-I–related (MR1) is a monomorphic β_2 microglobulin (β2m)–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 MHC molecules that constitutively bind to endogenous ligands, MR1 is predominantly sequestered in the endoplasmic reticulum (ER) in the steady state and 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, β2m 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 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, proinflammatory response against a diverse range of riboflavin-synthesizing pathogens [10–12].

While 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 type 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, while

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preexisting ligand-bound surface MR1 is protected from VZV-mediated targeting. Furthermore, we identify VZV open reading frame (ORF) 66 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.

MATERIALS AND METHODS

Cells

ARPE-19 MR1–green fluorescent protein (GFP) retinal pigment epithelial cells were engineered to overexpress MR1 with enhanced green fluorescent 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 [13]. Human embryonic kidney 293 (HEK 293T) cells were used for transfection experiments. All cells were maintained in Dulbecco's modified Eagle medium (Lonza) supplemented with 10% fetal calf serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco).

MR1 Ligands and Treatment

The synthetically derived MR1 ligand acetyl-6-formylpterin (Ac-6-FP) (Schirks Laboratories 29769-49-1) was added to media (5 μM) to induce MR1 surface expression [15]. 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 hours postinfection (hpi) or left untreated. Cells were harvested at 20 or 44 hpi. During transfection experiments, cells were treated with Ac-6-FP 24 hours posttransfection and harvested 28 hours posttransfection.

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) [16] were passaged in ARPE-19 cells. VZV-infected ARPE-19 cells at a cytopathic effect (CPE) of 3+ to 4+ (defined as 70%–100% of the monolayer showing CPE) were added to uninfected target cells at a ratio of 1:1 and incubated at 37°C 5% carbon dioxide.

Plasmid Constructs and Transfection

Plasmids pGK2-ORF66-HA and pGK2-ORF47-HA encode VZV ORF66 and ORF47, respectively, fused to an amino terminal hemagglutinin (HA) tag under the control of the HCMV immediate-early (IE) promoter. HEK 293T cells were transfected with 5 μg 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 previously [17]. 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–glycoprotein E:glycoprotein I (gE:gI) (clone SG1, Meridian Life Sciences) conjugated in-house to phycoerythrin (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 were analyzed using FlowJo software version 10.0.6. 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 Endoglycosidase 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 endoglycosidase H (Endo H; NEB) digested as per the manufacturer's instructions. Proteins were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Membranes were probed with anti-MR1 (number 55164, Abcam), anti-VZV IE62 (clone number IE62, Meridian) or anti-actin (number A2066, Sigma-Aldrich) followed by a horseradish peroxidase–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

While it is well-established that VZV downregulates MHC-I expression [18], the impact of this virus on 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,

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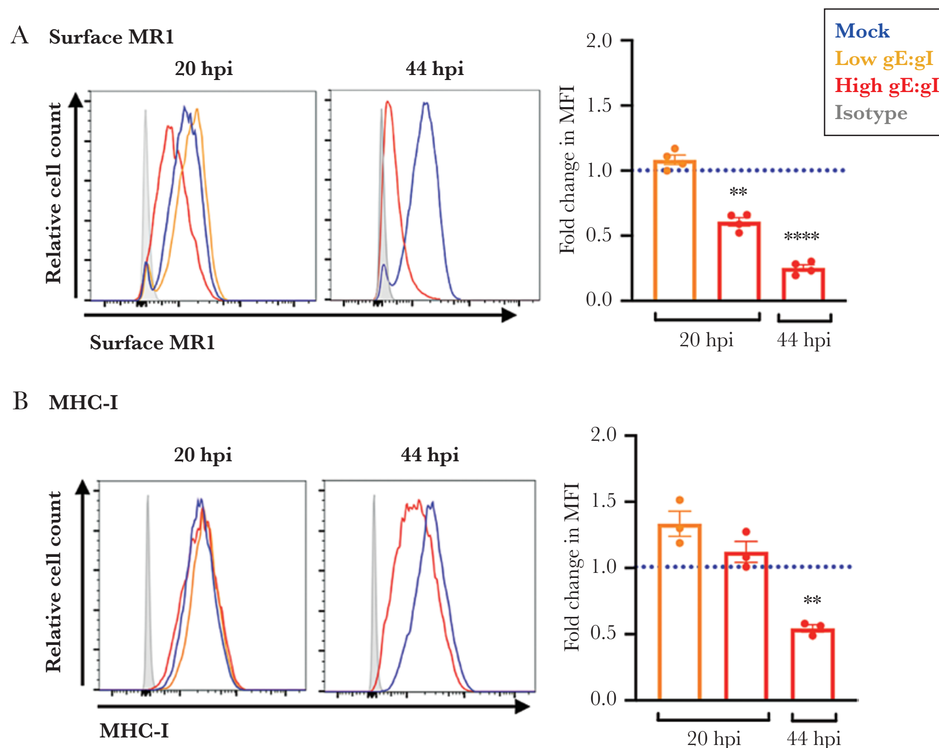

Figure 1. Varicella zoster virus (VZV) downregulates surface major histocompatibility complex, class I–related (MR1) at 20 and 44 hours postinfection (hpi). ARPE-19 MR1-GFP (*A*) or ARPE-19 (*B*) target cells were inoculated with VZV-S–infected ARPE-19 cells at a ratio of 1:1, or mock-inoculated in parallel. At 20 and 44 hpi cells were harvested, stained for cell-surface glycoprotein E:glycoprotein I (gE:gI), MR1 (*A*), major histocompatibility complex, class I (MHC-I; *B*) or isotype control antibody (gray histogram), and analyzed 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, and 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 standard error of the mean; significance compared to mock was calculated by Student *t* test. $^{**}P < .01$, $^{****}P < .0001$.

whereby CTV-labeled VZV-S–infected ARPE-19 cells (inoculum) were co-cultured for 20 or 44 hours with uninfected ARPE-19 MR1-GFP target cells [17] in a ligand-free assay. There was negligible cell death at both timepoints postinfection, 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 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 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 subpopulations permitted us to separately analyze 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 [19]. These findings indicate that VZV infection inhibits surface MR1 expression.

To confirm that VZV infection was unaffected by the MR1-GFP expression construct, CTV-labeled VZV-S–infected ARPE-19 cells (inoculum) were co-cultured 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 Pretreatment 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

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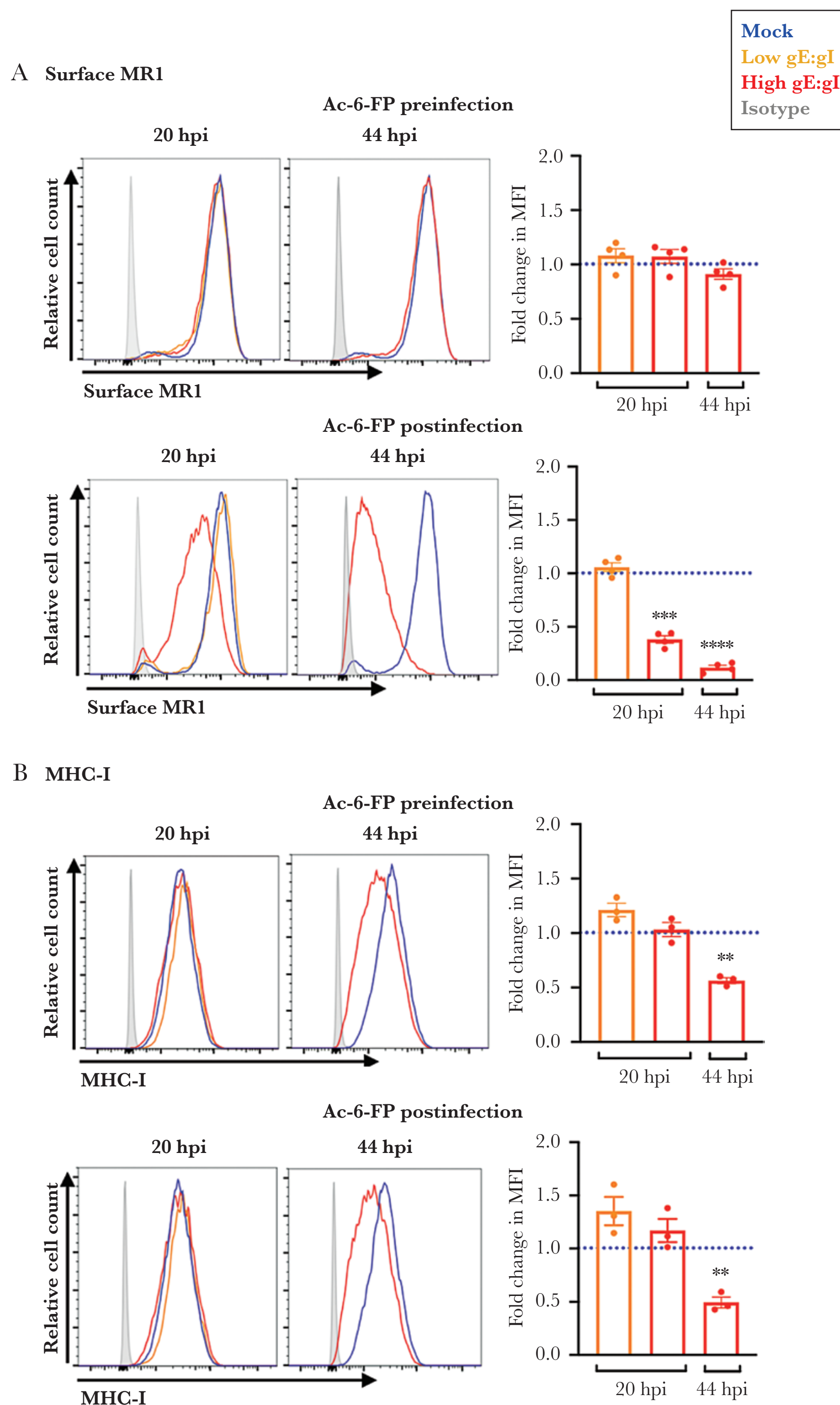

Figure 2. Ligand pretreatment protects surface major histocompatibility complex, class I–related (MR1) during varicella zoster virus (VZV) infection at 20 and 44 hours postinfection (hpi). ARPE-19 MR1-GFP (*A*) or ARPE-19 (*B*) target cells were inoculated with VZV-S–infected ARPE-19 cells at a ratio of 1:1, or mock-inoculated in parallel. Target cells were treated with acetyl-6-formylpterin (Ac-6-FP) ligand (5 μM) either 24 hours prior to VZV infection, or post–VZV infection at either 16 or 40 hpi (*A* and *B*). At 20 or 44 hpi, cells were harvested and stained for cell-surface glycoprotein E:glycoprotein I (gE:gI), MR1 (*A*), major histocompatibility complex, class I (MHC-I; *B*), or isotype control antibody (gray) and analyzed 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 median fluorescence intensity (MFI) fold change over mock was calculated for surface MR1 (n = 4) and MHC-I (n = 3). Error bars depict the standard error of the mean; significance compared to mock was calculated by Student *t* test. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

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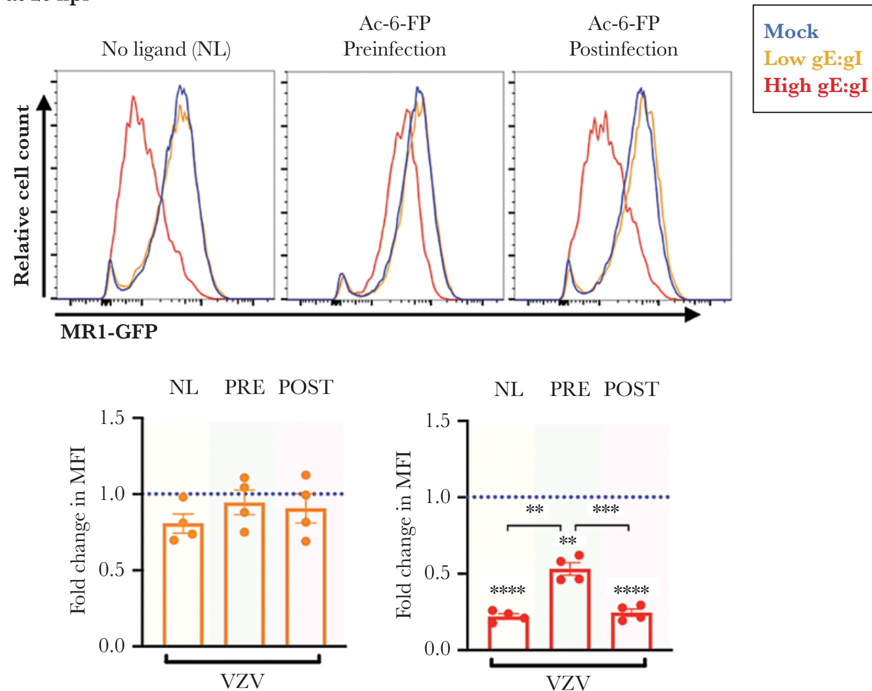

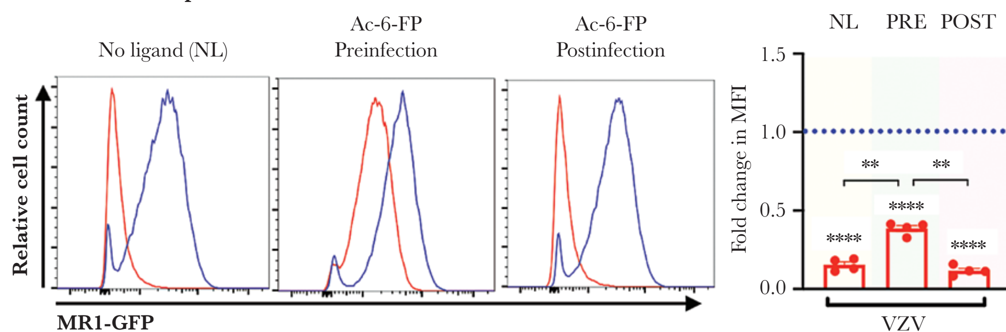

Figure 3. Varicella zoster virus (VZV) downregulates total major histocompatibility complex, class I–related (MR1)–GFP at 20 and 44 hours postinfection (hpi). ARPE-19 MR1-GFP cells were inoculated with VZV-S–infected ARPE-19 cells at a ratio of 1:1, or mock-inoculated in parallel. Target cells were treated with acetyl-6-formylpterin (Ac-6-FP) ligand (5 μM) 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 glycoprotein E:glycoprotein I (gE:gI), and analyzed 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 median fluorescence intensity (MFI) fold change over mock was calculated for total MR1 (n = 4). Error bars depict the standard error of the mean; significance compared to mock and between infected samples was calculated by Student *t* test. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

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 4 hours prior to staining. Surprisingly, cells pretreated 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 preexisting cell surface MR1 following treatment with ligand is protected from VZV targeting during infection. We have also observed that ligand pretreatment 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

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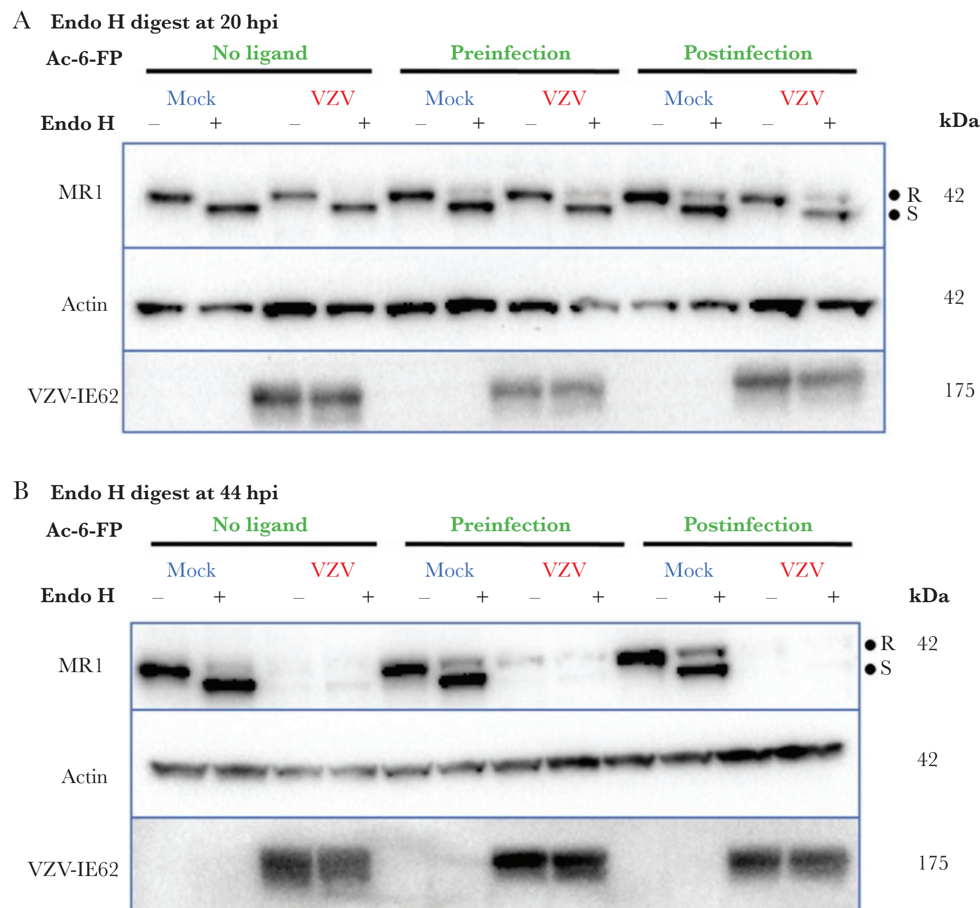

Figure 4. Mature endoglycosidase H (Endo H)–resistant major histocompatibility complex, class I–related (MR1) is retained during varicella zoster virus (VZV) infection. ARPE-19 MR1–overexpressing cells were infected with VZV-S–infected ARPE-19 cells at a ratio of 1:1, and mock-inoculated in parallel. Target cells were treated with acetyl-6-formylpterin (Ac-6-FP) ligand (5 μM) either 24 hours before or 16 (*A*) or 40 (*B*) hours post–VZV infection, or left untreated. At 20 (*A*) or 44 (*B*) hours postinfection (hpi), 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 2 biological repeats.

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 postinfection. 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 pretreatment of high gE:gI-expressing infected cells compared to those cells lacking ligand or receiving Ac-6-FP postinfection. 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 overexpress 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 (Figure 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

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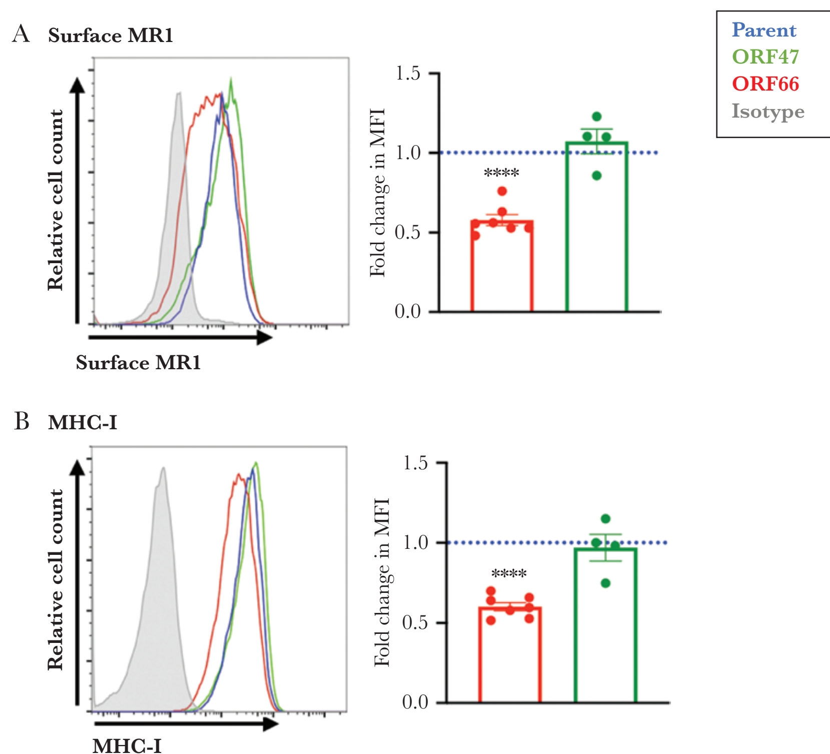

Figure 5. Varicella zoster virus open reading frame (ORF) 66 transient expression downregulates surface major histocompatibility complex, class I–related (MR1). HEK 293T cells were transfected with either ORF66 (n = 7) or ORF47 (n = 4) expressing plasmids (pGK2-HA-66 and pGK2-HA-47, respectively) or the parental (n = 7) control plasmid (pGK2-HA). Cells were treated with acetyl-6-formylpterin ligand (5 μM) 24 hours posttransfection. Cells were harvested at 28 hours posttransfection and stained for endogenous surface MR1, major histocompatibility complex, class I (MHC-I), intracellular hemagglutinin (HA), or isotype control (gray) and analyzed 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 median fluorescence intensity (MFI) fold change over parent control for surface MR1 (*A*) and MHC-I (*B*) was calculated. Error bars depict the standard error of the mean; significance compared to parent control was calculated by Student *t* test. ****$P < .0001$.

(Figure 4A and 4B). Infection of cells was validated by immunoblotting for VZV-encoded IE62 protein at 20 hpi and 44 hpi, and was probed 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 postinfection, whereas in cells pretreated 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). These data suggest 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 preexisting complex-glycosylated MR1.

VZV ORF66 Expression Partially Modulates Surface MR1 Expression

Given the involvement of VZV-encoded serine kinase ORF66 in downregulating MHC-I [18, 20], 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 [21].

HEK 293T cells were transfected with plasmids expressing HA-tagged ORF66 or ORF47 (pGK2-HA66 or pGK2-HA47, respectively) [22] 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) [18]. These data indicate that ORF66 impairs both surface MR1 and MHC-I, whereas ORF47 impacts neither cellular protein.

We next examined the capacity of 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 [16]. In ARPE-19 MR1-GFP target cells, both rOka and rOka 66s robustly downregulated surface MR1 in the absence and posttreatment (40 hpi) of ligand (Figure 6A). As demonstrated with VZV wild-type strain S infection (Figure

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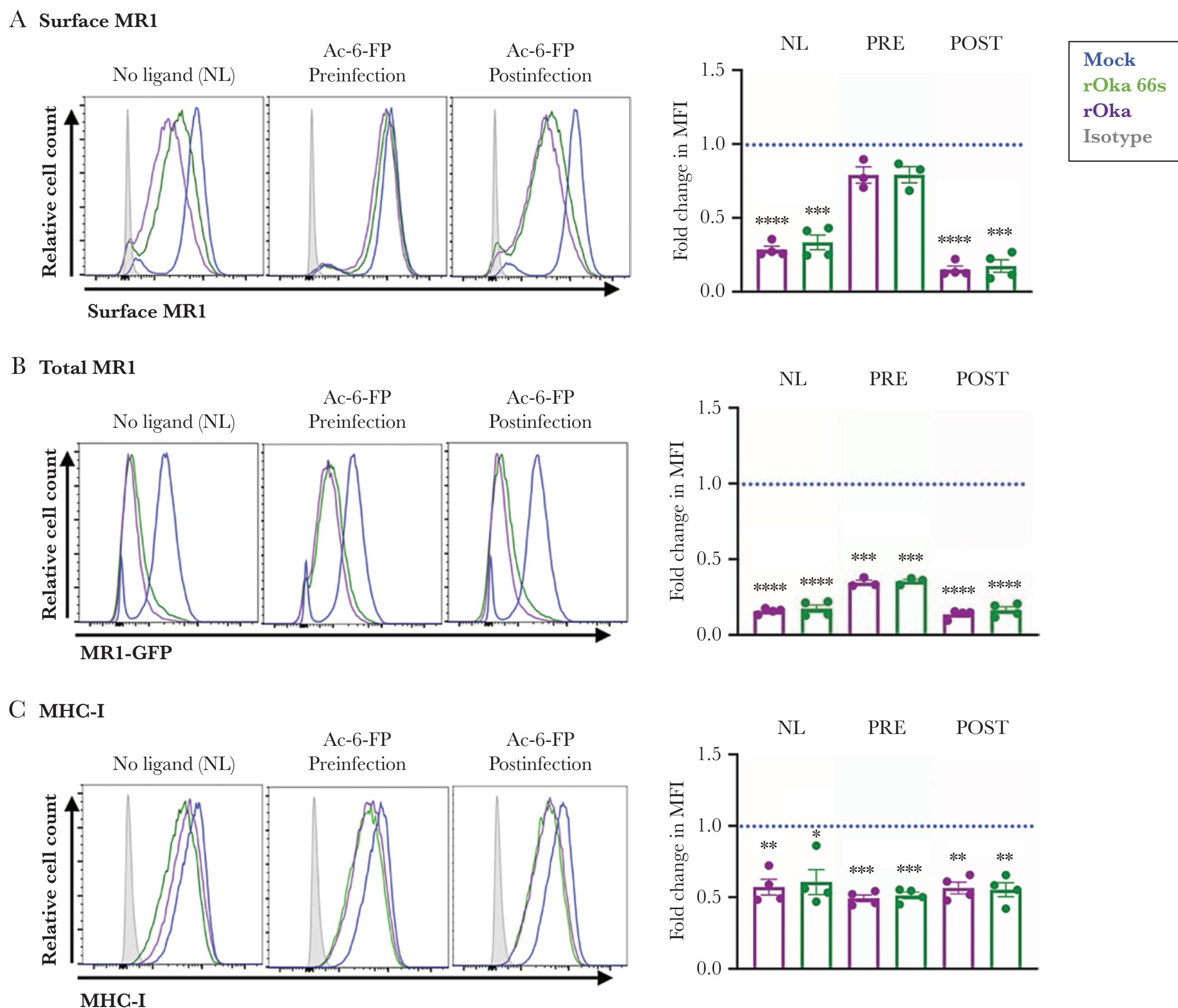

Figure 6. Varicella zoster virus (VZV) recombinant Oka strain (rOka) and rOka 66s similarly downregulate surface and total major histocompatibility complex, class I–related (MR1) at 44 hours postinfection (hpi). ARPE-19 MR1–GFP cells (*A* and *B*) or ARPE-19 cells (*C*) were inoculated with VZV rOka- or rOka 66s-infected ARPE-19 cells at a ratio of 1:1, or mock-inoculated in parallel. Target cells were treated with acetyl-6-formylpterin (Ac-6-FP) ligand (5 μM) either 24 hours prior to or after VZV infection at 40 hpi (*A*–*C*). At 44 hpi, cells were stained for surface glycoprotein E:glycoprotein I (gE:gI), surface MR1 (*A*), major histocompatibility complex, class I (MHC-I; *C*), or isotype control (gray) and analyzed 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. Median fluorescence intensity (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 standard error of the mean; significance against mock was calculated by Student *t* test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. NL indicates no ligand.

2A), surface MR1 was protected from downregulation in cells pretreated with ligand (24 hours preinfection) 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 [20]. While our data demonstrate 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, preexisting 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

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VZV-encoded serine/threonine kinase ORF66 contributing to MR1 downregulation in a transient gene expression setting, but also that it may not be the only contributor in the context of infection.

A common theme during primary VZV infection (varicella) is the high virus load at respiratory mucosal and skin sites. These sites are also enriched with resident MAIT cells, which respond to potential breaches in barrier integrity by detecting aberrant levels of metabolites from riboflavin synthesizing commensals [9, 23, 24]. During varicella, the production of vesicular lesions disrupts the skin and mucosal barriers, providing an opportunity for commensals to infect the host. The riboflavin pathway is expressed in a wide range of bacteria including *Staphylococcus aureus*, which is a common cause of secondary bacterial infections during varicella, ranging from the more frequently observed skin and soft tissue infections, to rare but serious visceral infections, sepsis, and toxic shock [25–29]. Consequently, during primary VZV infection, disruption to the mucosal barrier is likely to result in bacteria coinfecting or colocating at sites of varicella lesions, providing sources of MR1 ligands. Thus, VZV modulation of MR1 antigen presentation may impact MR1-dependent presentation of intracellular- or extracellular-derived ligands to MAIT cells. First, given that MR1-TCR mediated activation of MAIT cells precedes its response to cytokines [30], MAIT cell activation could be delayed. In addition, it may avert the establishment of the immunological synapse between antigen-bound surface MR1 and the MAIT TCR that directs the targeted cytolytic killing [10] of infected cells. Finally, it may prevent the synergistic effect of TCR-mediated and innate signaling that is required for sustained activation and proliferation of MAIT cells [31–33]. These combined impacts on MAIT cell activation and effector responses could thus provide critical time for optimal viral replication and transmission to new host cells. However, whether the modulation of MR1 by VZV provides a benefit to this virus requires further examination, and functional studies of MAIT cell responsiveness using models of VZV and bacterial coinfections will be important to define 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 [34, 35]. In this respect, the involvement of MAIT cells in various viral infections such as influenza virus, hepatitis B virus (HBV), hepatitis C virus, and human immunodeficiency virus has become apparent, with studies reporting altered circulating MAIT cell frequencies and functions [36–38]. In contrast, evidence exploring the role of MR1-dependent antigen presentation during viral infection remains limited. We previously reported that epithelial cells pretreated 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 [39]. In addition, severe acute respiratory syndrome coronavirus 2–infected macrophages trigger MR1-dependent MAIT cell granzyme B production and degranulation in vitro [40]. Finally, MR1 trafficking has been shown to be dependent on NF-κB signaling [34], a pathway that is critical to establishing the antiviral response and is actively modulated by herpesviruses such as VZV [41]. 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 preexisting mature ligand-bound surface MR1, which is 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 4 gene products that each target various steps within the MHC-I antigen presentation pathway [42]. During VZV, but not HSV-1 infection, MHC-I molecules accumulate in the Golgi [18]. On the other hand, while HSV-1 ICP47 blocks peptide transport into the ER lumen [43], a functional homolog of this immunomodulatory gene is lacking in the VZV genome. Transient gene expression revealed that ORF66 (Figure 5) impacts MR1 in the absence of other viral gene products, whereas the ORF66s mutant failed to rescue surface MR1 or MHC-I (Figure 6), implying that other VZV genes play important roles in the modulation of these immune molecules during viral infection.

ORF66 has orthologs in all alphaherpesviruses and is considered part of the US3 family of herpesvirus protein kinases. They have common features in that their targets are basophilic [44] and sometimes overlap with the basophilic cellular protein kinase PKA [45]. While many HSV-1 Us3 targets have been identified, only a small number have been confirmed for VZV ORF66, including the cellular nuclear matrix protein Matrin 3, type 1 histone deacetylases, and viral protein IE62 [22, 45, 46]. Until more targets are identified, the predicted consensus sequence for ORF66 remains loosely defined, and thus at present narrowing the list of potential cellular targets involved in MR1 downregulation remains a challenge.

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

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

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Notes

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

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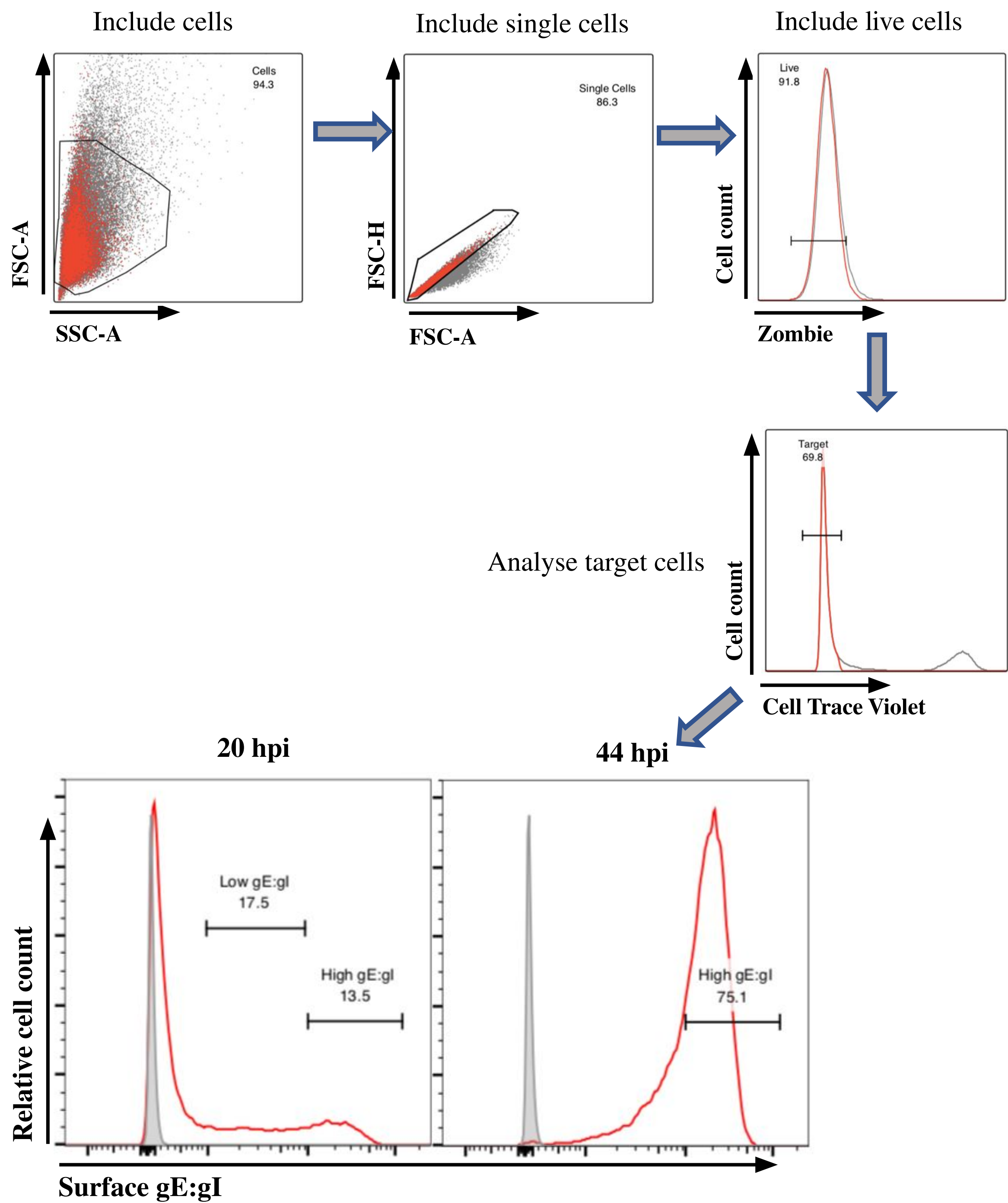

Supplementary Figure 1. Flow cytometry gating strategy to evaluate VZV infected target cells

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

Chapter 3. Infection of Mucosal Associated Invariant T cells by Varicella Zoster Virus

3.1 Introductory statement

Previous investigations have established VZV to be a highly lymphotropic virus that infects diverse immune populations. In particular, VZV effectively induces skin trafficking programs in mature T cells as well as circulating NK cells (as reviewed in Chapter 1.3.2). Given the circulating abundance and inherent predilection of MAIT cells to traffic to mucosal and skin sites, we hypothesised MAIT cells to be a pathogenically relevant target for VZV infection.

Therefore, we investigated the capacity for VZV to infect MAIT cells. The findings generated from this study were published in Frontiers in Immunology in 2023 and forms Chapter 3 of this thesis. The materials and methods used for generating the data within this publication are all enclosed as a subsection within the paper and not a separate thesis chapter. Similarly, all literature citations and figures associated with supplementary information are included as subsections within the publication.

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Varicella Zoster Virus infects mucosal associated Invariant T cells

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Introduction: Mucosal Associated Invariant T (MAIT) cells are innate-like T cells that respond to conserved pathogen-derived vitamin B metabolites presented by the MHC class I related-1 molecule (MR1) antigen presentation pathway. Whilst viruses do not synthesize these metabolites, we have reported that varicella zoster virus (VZV) profoundly suppresses MR1 expression, implicating this virus in manipulation of the MR1:MAIT cell axis. During primary infection, the lymphotropism of VZV is likely to be instrumental in hematogenous dissemination of virus to gain access to cutaneous sites where it clinically manifests as varicella (chickenpox). However, MAIT cells, which are found in the blood and at mucosal and other organ sites, have yet to be examined in the context of VZV infection. The goal of this study was to examine any direct impact of VZV on MAIT cells.

Methods: Using flow cytometry, we interrogated whether primary blood derived MAIT cells are permissive to infection by VZV whilst further analysing differential levels of infection between various MAIT cell subpopulations. Changes in cell surface extravasation, skin homing, activation and proliferation markers after VZV infection of MAIT cells was also assessed via flow cytometry. Finally the capacity of MAIT cells to transfer infectious virus was tested through an infectious center assay and imaged via fluorescence microscopy.

Results: We identify primary blood-derived MAIT cells as being permissive to VZV infection. A consequence of VZV infection of MAIT cells was their capacity to transfer infectious virus to other permissive cells, consistent with MAIT cells supporting productive infection. When subgrouping MAIT cells by their co- expression of a variety cell surface markers, there was a higher proportion of VZV infected MAIT cells co-expressing CD4+ and CD4+/CD8+ MAIT cells compared to the more phenotypically dominant CD8+ MAIT cells, whereas infection was not associated with differences in co-expression of CD56 (MAIT cell subset with enhanced responsiveness to innate cytokine stimulation), CD27 (co-stimulatory) or PD-1 (immune checkpoint). Infected MAIT cells retained high expression of CCR2, CCR5, CCR6, CLA and CCR4, indicating a potentially intact capacity for transendothelial migration, extravasation and trafficking to skin sites. Infected MAIT cells also displayed increased expression of CD69 (early activation) and CD71 (proliferation) markers.

Discussion: These data identify MAIT cells as being permissive to VZV infection and identify impacts of such infection on co- expressed functional markers.

KEYWORDS

Varicella Zoster Virus, productive infection, MAIT cells, herpesvirus, innate-like T cells

Introduction

Compared to conventional T lymphocytes that can respond to a very wide array of peptide antigens, innate T cells react to conserved antigenic patterns either pathogenically or host derived. Mucosal Associated Invariant T (MAIT) cells are one of the largest human innate T cell subpopulations, representing approximately 3% of all circulating T cells and up to 45% of liver T cells in healthy donors (1, 2). MAIT cells express a limited semi-invariant T cell receptor (TCR) repertoire allowing for their recognition of microbial metabolite neo-antigens presented by the monomorphic MHC- related 1 (MR1) molecule (3–6). The most well characterized MAIT cell agonist ligand, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), is a pyrimidine derivate from the vitamin B2 (riboflavin) biosynthesis pathway presented by MR1 (5, 7). The conservation of this biosynthetic pathway across a diverse range of bacterial and fungal species drives evolutionary conservation of the MR1-MAIT cell axis within mammals (8).

Given the inherent barrier surveillance functionality of MAIT cells, they are tailored to have a highly expressed extravasation program mediated by C-C Chemokine receptor (CCR)2, CCR5 and CCR6 expression (9), complimented by their migrational predilection to anatomical sites such as liver, respiratory mucosa and skin mediated by expression of CCR6, CCR5, CCR9, C-X-C Chemokine receptor (CXCR)6 and cutaneous lymphocyte antigen (CLA) (1, 2, 10, 11). Whilst MAIT cells can rapidly react to their cognate ligands, the concomitant delivery of accessory signals such as Toll-Like receptors (TLRs) and cytokines such IL-12 and IL-18 elicit a broader and more sustained effector response (12–16). Indeed, MAIT cells can be solely activated by cytokines in the absence of TCR engagement driving a distinct effector response (17). Co-incubation of MAIT cells with intact riboflavin synthesizing bacteria rapidly stimulates a Tc17-like RORγt driven response characterized by granzyme B mediated cytolytic activity, proinflammatory cytokine expression of IFN-γ, TNF-α, IL-17α, as well as tissue repair signals such as TGF-β and furin (16, 18–20). Comparatively, IL-12 and IL-18 driven activation of MAIT cells is delayed and Tc1-like as characterized by increased T-bet, IFN-γ and granzyme B expression (20). Combined, both modes of MAIT cells activation support a polyfunctional T cell population that is able to enact diverse and distinct effector responses dependent on the micro-environmental cues.

Cytokine driven activation of MAIT cells has stimulated a burgeoning interest in the importance of MAIT cells in controlling viral infections. Whilst a growing body of literature has reported a protective role of MAIT cells in several viral infections both *in vitro* and *in vivo* (21–25), there is a dearth of studies that examines virus infection of MAIT cells. To date, there is only a single study reporting virus infection of MAIT cells; infection and apoptosis of MAIT cells *in vitro* by measles virus (MV) (26).

Varicella Zoster Virus (VZV) is a lymphotropic, highly seroprevalent alpha herpesvirus that causes varicella during primary infection and herpes zoster following reactivation from latency (27). Following exposure to infected respiratory droplets, VZV initially infects the epithelial cells and resident dendritic cells (DCs) lining the upper respiratory tract before gaining access to local lymphoid structures such as the tonsils (28). Here, the transfer of virus is believed to occur from DCs to mature T lymphocytes (28, 29) that express skin homing markers, with several reports characterizing how this enables VZV to reach the host's cutaneous sites (30–34). We reported that VZV productively infects human natural killer (NK) cells *in vitro*, resulting in their upregulation of skin homing capacity, yet overall functional paralysis (35, 36). These findings suggest that VZV has evolved the capacity to infect a broad range of immune cell types to enhance virus dissemination.

We have previously demonstrated a profound disruption of the MR1 antigen presentation pathway mediated by VZV, thereby suggesting a virally pathogenic importance in abrogating the TCR dependent activation and effector response of MAIT cells (37). However, the direct infection or interaction of VZV with MAIT cells themselves had yet to be investigated, despite the substantial enrichment of MAIT cells in blood as well as migrational proclivity to tissues central to VZV pathogenesis such as airway epithelia and skin. In the current study, we examined the ability of VZV to infect human blood-derived MAIT cells *in vitro*. We demonstrate that VZV productively infects MAIT cells, resulting in a capacity to transmit virus to other cells. VZV infection of MAIT cells was associated with retention or upregulation of markers of extravasation, skin homing potential and/or activation and proliferation. Overall, this study illuminates an innate-like T cell population that can be directly targeted by VZV.

Materials and methods

Blood samples and MAIT cell isolation

Healthy adult human donor buffy coats were obtained from Australian Red Cross Lifeblood service from which peripheral blood mononuclear cells (PBMCs) were isolated through density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). There was no selection bias based on gender. Isolated PBMCs were cultured in complete RPMI medium (RPMI 1640 with L-Glutamine (Lonza) supplemented with 10% human serum (Sigma-Aldrich). In experiments which utilized purified MAIT cells, MAIT cells were FACS isolated from PBMCs after positive co-staining in FACS buffer (PBS supplemented with 1% FCS and 10 mM EDTA) with fluorochrome conjugated antibodies: anti-CD3, anti-TCR Vα7.2 and 5-OP-RU loaded MR1-Tetramer. MAIT cells were sorted to a >98% purity using BD Influx (BD Biosciences). For some experiments, where specified, MAIT cells were further FACS sorted on the basis of CD69 expression to a purity of >97% for both $CD69^-$ and $CD69^+$ populations using BD Influx (BD Biosciences).

Cell culture and viruses

ARPE-19 epithelial cells (ATCC) and ARPE-19-GFP cells that overexpress MR1 with GFP under the same promoter *via* a

downstream internal ribosome entry sequence (38) were cultured in complete DMEM medium (DMEM with 4.5 g/L glucose and L-glutamine (Lonza), supplemented with 10% Foetal calf serum (FCS) (Sigma Aldrich) and 1% penicillin streptomycin (Gibco). A clinical VZV strain (VZV-S) and a recombinant VZV rOka-ORF10-GFP (VZV-GFP), which expresses GFP in fusion with ORF10 (39), were propagated in ARPE-19 cells in complete DMEM medium. All cells were cultured at 37 °C 5% CO_2.

VZV infection of immune cells and epithelial cells

PBMCs were either mock or VZV inoculated *via* co-culture with either uninfected or VZV infected ARPE-19 cells, respectively. The viral inoculum consisted of >75% VZV infected ARPE-19 cells demonstrating cytopathic effect (CPE). Inoculum was trypsinized, washed and resuspended in supplemented RPMI and added to PBMCs at a ratio of 1:2-5 ARPE-19: PBMC. For some experiments assessing viral infectivity, inoculum was added to PBMCs at various ratios of: 1:2, 1:5, 1:10, and 1:20 inoculum: PBMC. In parallel, inoculum was also added to ARPE-19 GFP expressing cells at identical ratios of 1:2, 1:5, 1:10 and 1:20 inoculum: ARPE-19-GFP cells for comparison. Kinetics of infection was investigated *via* a time-course experiment, inoculum was co-cultured with either PBMCs or ARPE-19-GFP cells at a ratio of 1:5 and harvested at various time-points of: 6, 24, 48 and 72 hours post inoculation. For experiments using total PBMCs, infections were performed in 12-well plates with 1-2 x 10^6 PBMCs in 2 ml of complete RPMI medium per well. For experiments using FACS sorted MAIT cells, infections were performed in 24-well plates with 4 x 10^5 cells in 600 ml complete RPMI medium per well. Following the addition of either mock or VZV infected cells to sorted MAIT cells and/or total PBMCs, cells were spinoculated in tissue culture plates for 15 minutes at 150 x *g* at 37°C. Plates were then incubated at 37°C 5% CO_2 for 2 days.

Antibodies

PBMCs for either surface staining flow cytometry or FACS sorting experiments were stained with the following fluorochrome conjugated antibodies: CD3-BUV395 (SK7), CD25-APC-H7 (M-A251), CD27-BUV661 (M-T271) (all BD Bioscience), CD8-SB780 (OKT8) (Thermo Fisher), CD4-PerCP/Cy5.5 (OKT4), CD56-BV605 (NCAM 16.2), TCR Vα7.2 (OF5A12), CCR4-BV421 (L291H4), CLA-AF647 (HECA-452), CCR2-APC (K036C2), CCR5-PE/Cy7 (J418F1), CCR6-BV421 (G034E3), CD69-BV421 (FN50), PD-1-PE/Dazzle (EH12.2H7), CD71-BV650 (CY1G4) (all Biolegend), VZV gE:gI (SG1-1, conjugated in house to Dy488), VZV gE:gI (SG1-1, conjugated in house to PE) (Meridian Life Sciences), 5-OP-RU loaded MR1 tetramer-PE, Ac-6-FP loaded MR1 Tetramer-PE. Matched isotype controls were used as negative controls.

Flow cytometry

Cells were collected and viability stained with Live/Dead Blue (Invitrogen) as per manufacturer's protocol. Cells were then resuspended and washed in FACS buffer, before staining with antibodies on ice for 45 minutes. Cells were washed in FACS buffer then fixed in 4.2% formaldehyde (BD Biosciences) at 4°C for 15 minutes before acquiring on a LSR-II cytometer (BD Biosciences).

Flow cytometry data analysis

Data was analyzed using FlowJo software (versions 10.0.7 and 10.2; Tree Star). All PBMC data depicted was gated on live (as per the Live/Dead Blue viability dye staining) lymphocytes (as per distinct forward and side scatter morphology). MAIT cells were identified through positive co-staining with 5-OP-RU loaded MR1-Tetramer, anti-CD3 and anti-Vα7.2. All data observing viral infection of ARPE-19-GFP cells was gated on live (as per the Live/Dead Blue viability dye staining) GFP expressing cells.

Infectious center assay

PBMCs were co-cultured with mock or VZV-ORF10-GFP infected ARPE-19 cells for 2 days. Cells were collected, stained and FACS sorted for $CD3^+$ MR1-$Tetramer^+$ $V\alpha7.2^+$ (MAIT) cells. To remove extracellular virus, MAIT cells were washed in citrate buffer (40 mM C6H5O7Na3, 135 mM NaCl, 10 mM KCl [pH 3]), at room temperature for 2 minutes before washing in PBS (35, 40–42). In duplicate, MAIT cells ($2.5x10^5$) were resuspended in complete RPMI medium and then added to pre-seeded ARPE-19 monolayers ($7.5x10^5$) on glass coverslips in 24-well plates. Co-cultures were spinoculated at 15 minutes at 150 x *g* 37°C, before being incubated at 37°C 5% CO_2 for 5 days to allow for formation of any CPE. Monolayers were fixed with 4.2% formaldehyde (BD Biosciences) at room temperature for 15 minutes, and then counterstained with DAPI. Imaging was performed using the Nikon Ti2-E Widefield fluorescence microscope.

Statistical analyses

Statistical analyses were performed using GraphPad Prism (version 9; GraphPad Software).

Ethics statement

All blood work was performed in accordance with The University of Sydney Human Research Ethics Committee approval. All blood donations were obtained under agreement with the Australian Red Cross Lifeblood service.

Results

Varicella Zoster Virus infects primary MAIT cells in human peripheral blood

Whilst the ability of VZV to infect human T cells is well documented (30, 31, 33, 34), the permissibility of MAIT cells to VZV is not known. To investigate this potential interaction, we assessed *via* flow cytometry the capacity of VZV clinical isolate (VZV-S) infected ARPE-19 epithelial cell-associated inoculum to infect MAIT cells in PBMCs. This cell-associated model of infection is commonly used in VZV studies (29, 30, 35) as VZV is very highly cell-associated *in vitro* (43). The mock and VZV infected inocula were excluded from analysis as per FSC-A SSC-A morphology gating and CD3 negative expression (Figure 1). The detection of the surface VZV glycoprotein (g)E:gI complex, which is expressed late in the VZV replicative cycle, was utilized as a means to detect virally infected cells (33, 35–37, 44, 45). Therefore, all results presented within this study that refer to the "VZV^{+}" or "VZV infected" are expressing VZV gE:gI.

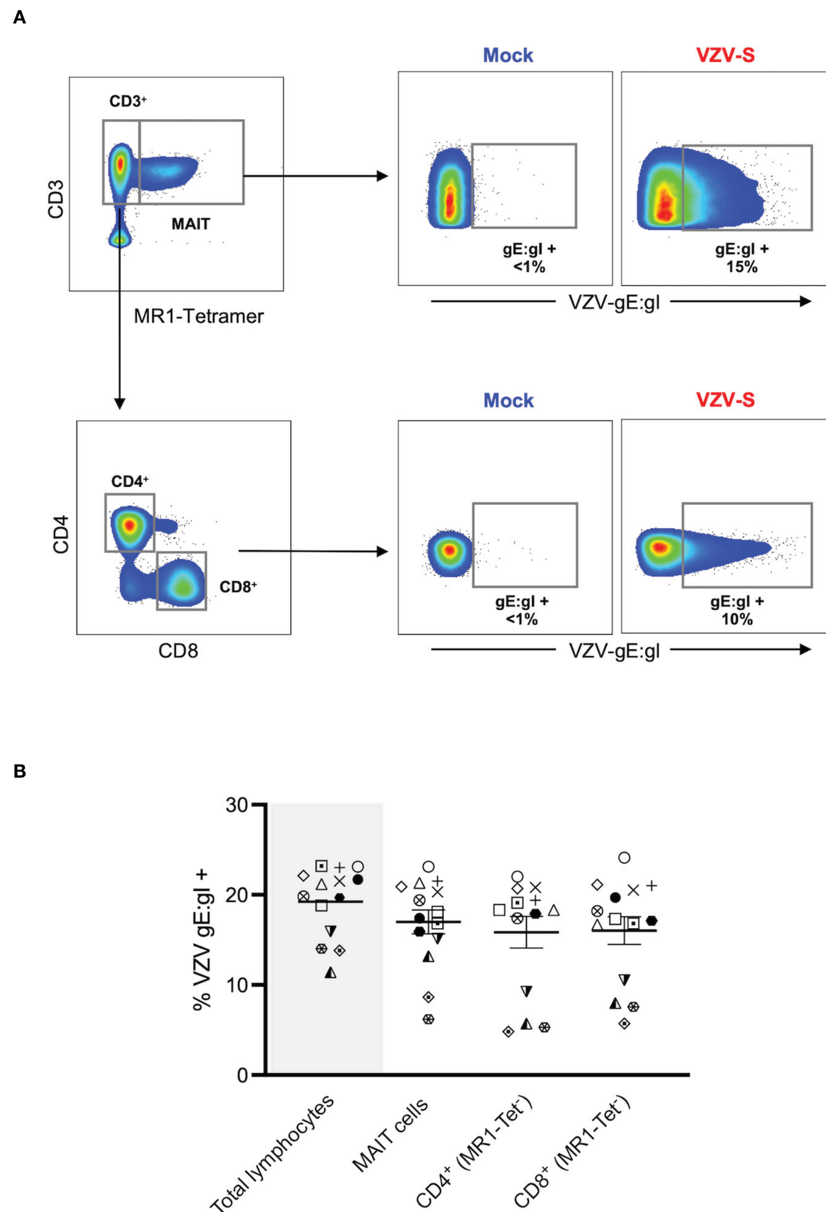

FIGURE 1
VZV infects MAIT cells from human peripheral blood. Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for 2 days and then analyzed for infection by flow cytometry. **(A)** Representative flow cytometry plots depicting gating strategy of CD3^{+} MR1-Tetramer^{+} (MR1-Tet^{+}) MAIT cells, non-MAIT (ie MR1-Tet^{-}) CD3^{+} CD4^{+} (CD4 T cells) and non-MAIT (ie MR1-Tet^{-}) CD3^{+} CD8^{+} cells (CD8 T cells), as well as quantifying surface VZV glycoprotein (g)E:gI expression on gated populations. **(B)** Frequencies of total live gE:gI^{+} lymphocytes (shaded), compared to MAIT cells, non-MAIT CD4^{+} and non-MAIT CD8^{+} cells (n=14). Symbols represent individual donors across the lymphocyte populations, with mean and standard error of mean (SEM) indicated by the bars. Statistical analysis between gE:gI expression on specific lymphocyte populations was performed *via* repeated measures (RM) one-way ANOVA with the Greenhouse-Geisser correction and Tukey's multiple comparisons test.

MAIT cells were identified *via* co-staining of 5-OP-RU loaded MR1 tetramer and CD3 (Figure 1A), with a vast majority of that population positively staining for the canonical MAIT TCR, Vα7.2 (range: 96.2-99.8%) (Supplementary Figure 1A). In line with previous studies (1, 2), we detected an average of 2.4% of live PBMCs as MAIT cells, with no difference in the frequency of live MAIT cells between mock and VZV inoculated samples (Supplementary Figure 1B). When examining the transfer of viral infection to the total pool of live PBMCs, a mean of 19.2% (range 11.4-23.2%) of these cells were VZV gE:gI$^+$ (Figure 1B).

A mean of 17% of MAIT cells were gE:gI$^+$ (range 6.2-23.2%). When examining gE:gI expression in non-MAIT cell (ie MR1 Tet$^-$) CD4$^+$ cells (mean 15.8%, range 4.8-22%) and non-MAIT cell (ie MR1-Tet$^-$) CD8$^+$ cells (mean 16%, range 5.7-24.1%) populations, we observed no significant difference in infection level when compared to MAIT cells across 14 different donors (Figure 1B). Furthermore, gE:gI expression on MAIT cells was detected as early as 6 hours post inoculation (mean 4.7%, range 3.6-5.2%) and peaked at 48 hours post inoculation (mean 23%, range 20.5-25%) (Supplementary Figure 2A). Additionally, gE:gI expression was detected on MAIT cells at various viral inoculum: PBMC ratios from 1:2 (mean 21.7%, range 20.5-22.6%) to 1:20 (mean 7.7%, range 5-12.1%) (Supplementary Figure 2B). In a comparison to epithelial (ARPE-19) cells, MAIT cells were less permissive to VZV infection (Supplementary Figure 2). Together, these results identify MAIT cells as a T lymphocyte compartment that is permissive to VZV infection. Furthermore, VZV infection of MAIT cells was less than that of infection of ARPE-19 cells but comparable to infection of non-MAIT cell CD4$^+$ and CD8$^+$ T lymphocyte populations.

VZV infects diverse MAIT cell subsets

Similar to other innate-like T cell populations, the MAIT cell compartment consists of a heterogenous mix of subpopulations with distinct functional attributes (1, 46, 47). Using flow cytometry, we sought to determine the extent to which VZV infection of MAIT cells was associated with different subsets of MAIT cells. MAIT cells were split into distinct subpopulations based on the expression of the following cell surface markers: co-receptor (CD8 and CD4), co-stimulatory (CD27), immune checkpoint marker (PD-1), and CD56. Flow cytometric analysis revealed the frequencies of MAIT cell sub-populations (Figure 2A), and these were consistent with previous literature (1, 46–48). Furthermore, no change of frequencies within MAIT cell subpopulations between mock and VZV infected samples was observed (Supplementary Figure 1C).

When examining the co-receptor subpopulations, there was a significantly higher level of infection of CD4$^+$ (mean 23.6%, range 12-32.5%) and double positive (CD4$^+$/CD8$^+$) (mean 22.8%, range 9.6-35.1%) MAIT cells compared to CD8$^+$ (average 16.8%, range 6.2-22.7%) and double negative (CD4$^-$/CD8$^-$) (mean 16%, range 5.4- 31.4%) MAIT cells (Figure 2B). There was no significant difference in the level of infection between CD56$^+$ (mean 18.1%, range 6.6-30.6%) and CD56$^-$ (mean 17.1%, range 6.1-23.4%) MAIT cells (Figure 2B). Furthermore, there was no significant difference in the level of infection between CD27$^+$ (mean 7.9%, range 6.2-10.4%) and CD27$^-$ (mean 9.4%, range 6.3-15%) MAIT cells, and both PD-1$^+$ (mean 9.3%, range 6.5-14.5%) and PD-1$^-$ (mean 7.7%, range 6.1-10.2%) MAIT cells demonstrated similar levels of infection (Figure 2B). Overall, all subpopulations examined in this study were comparably infected, with the exception of the proportion of CD4$^+$ and CD8$^+$/CD4$^+$ double positive MAIT cells being associated most with VZV infection.

VZV infection of MAIT cells is associated with increased CD69 and CD71 expression

We sought to determine whether VZV infection of MAIT cells was associated with an altered expression profile of activation (CD69) and proliferation (CD71) markers, as determined by flow cytometry of mock and VZV infected MAIT cells. As an additional comparison we also examined mock and VZV infection of non-MAIT (ie MR1-Tet$^-$) CD3$^+$ T cells. Mock inoculated MAIT cells endogenously expressed higher CD71 (mean 1%, SEM +/- 0.15%) compared to non-MAIT CD3$^+$ T cells (mean 2.18%, SEM +/- 0.19%). Whilst a significantly greater proportion of mock MAIT cells endogenously expressed higher CD69 (mean 1.95%, SEM +/- 0.35%) compared to non-MAIT CD3$^+$ T cells (mean 19.95%, SEM +/- 3.56%) (Figure 3A). Furthermore, our analysis revealed a significantly higher proportion of VZV infected (gE:gI$^+$) MAIT cells expressed CD71 (mean 40.62, SEM +/- 2.01) compared to mock infected (mean 2.18%, SEM +/- 0.19%) (Figure 3B). This was also the case when observing CD69 expression in VZV infected MAIT cells (mean 36.15%, SEM +/- 6.32%) compared to mock (mean 19.95%, SEM +/- 3.56%) (Figure 3B). Indeed, a significantly greater number of CD71/CD69 double positive MAIT cells was correspondingly observed in the VZV infected condition (mean 15.84%, SEM +/- 2.59%) compared to mock (mean 0.99%, SEM +/- 0.17%) (Figure 3C). Furthermore, a higher proportion of CD69 and CD71 expressing cells were similarly detected in VZV infected non-MAIT CD3$^+$ T cells compared to mock infected counterparts (Figure 3B). When analysing the VZV bystander (gE:gI$^-$) MAIT cell subpopulation, only a conservative albeit significant increase of CD71 expression compared to mock MAIT cells was observed (mean 4.58%, SEM +/- 0.1%), whilst no significant change of CD69 expression compared to mock was detected (Supplementary Figure 4A).

Following on previous reports that demonstrate a preferential infection of CD69 expressing T lymphocytes (30) we FACS sorted MAIT cells by CD69 expression into two populations: CD69$^-$ and CD69$^+$ MAIT cells (Supplementary Figure 3A). We observed no significant difference in VZV infection when comparing CD69$^-$ and CD69$^+$ sorted MAIT cells (Supplementary Figure 3B). We also examined CD69 expression on these MAIT cells sorted on the basis of CD69 expression. In comparison to mock infected counterparts, we did not observe an increase of CD69 expression by CD69-sorted MAIT cells following VZV infection, however, there was a significant increase in CD69 expression following VZV infection of CD69$^+$ sorted MAIT cells (Supplementary Figure 3C). Collectively, these data indicate that VZV infection of MAIT cells and non-MAIT T cells is associated with the upregulation of CD71$^+$ and CD69$^+$ expression.

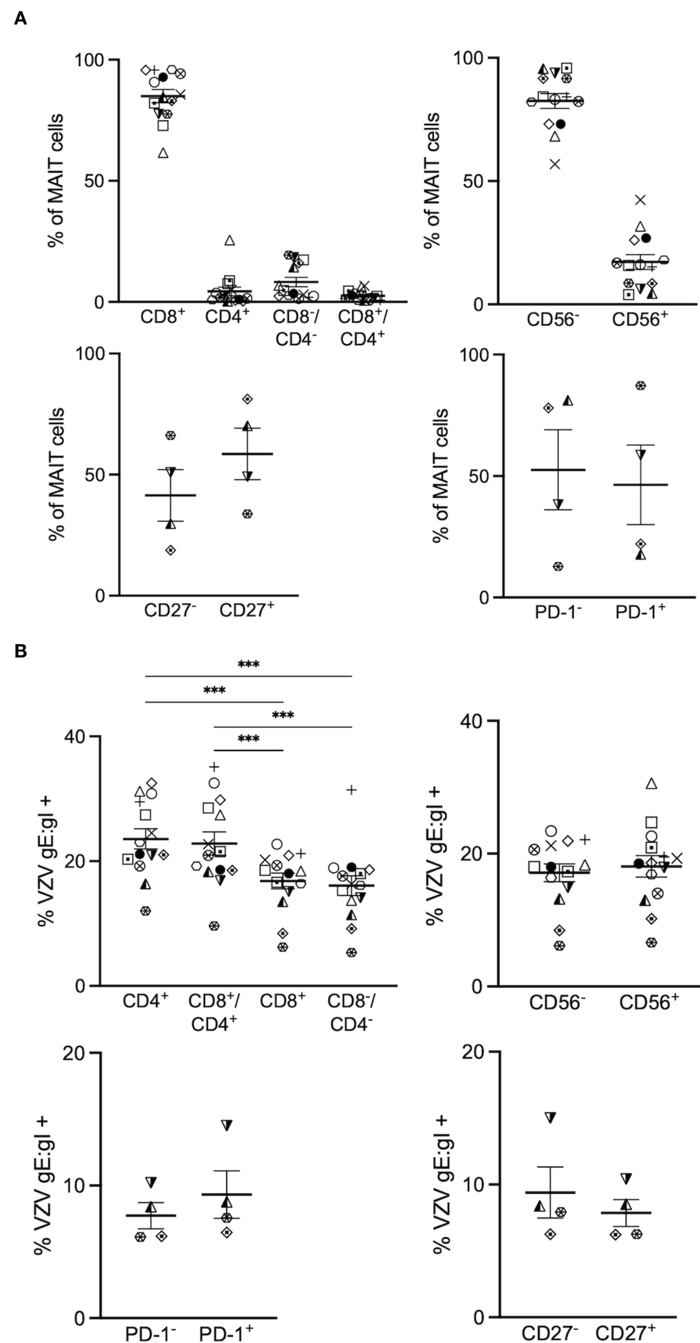

FIGURE 2
VZV infects diverse MAIT cell subsets. Human PBMCs were inoculated with mock or VZV-S infected ARPE-19 epithelial cells for 2 days and then analyzed for infection by flow cytometry as per surface VZV-gE:gI expression **(A)** Graphs showing frequencies of various MAIT cell subpopulations (n=4-14). **(B)** Frequencies of gE:gI^{+} lymphocytes within each subpopulation depicted, with symbols representing individual donors across the MAIT subpopulations, with mean and SEM indicated by the bars. Statistical analysis of VZV gE:gI expression, comparing CD4^{+} cells with CD8^{+} cells, and CD4^{+}/CD8^{+} cells compared to CD4^{-}/CD8^{-} cells was performed *via* RM one-way ANOVA with the Greenhouse-Geisser correction and Tukey's multiple comparisons test (n=14).***p<0.001. Statistical analysis of gE:gI expression on MAIT cells expressing CD56^{-} was compared to those expressing CD56^{+} (n=14), CD27^{-} compared to CD27^{+} (n=4) and PD-1^{-} compared to PD-1^{+} (n=4) was performed *via* two tailed paired *t* test.

VZV infected MAIT cells maintain a highly expressed extravasation and skin homing program

We next sought to determine the impact of VZV infection on the natively high expression of extravasation and skin homing markers on MAIT cells (9, 10). Initially, expression of key extravasation markers CCR2, CCR5 and CCR6 on mock inoculated non-MAIT (ie MR1-Tet^{-}) CD3^{+} cells was compared to mock infected MAIT cells. This analysis revealed that a greater proportion of MAIT cells endogenously expressed CCR5, CCR6 and CCR2 (Figure 4A), which is consistent with MAIT cells

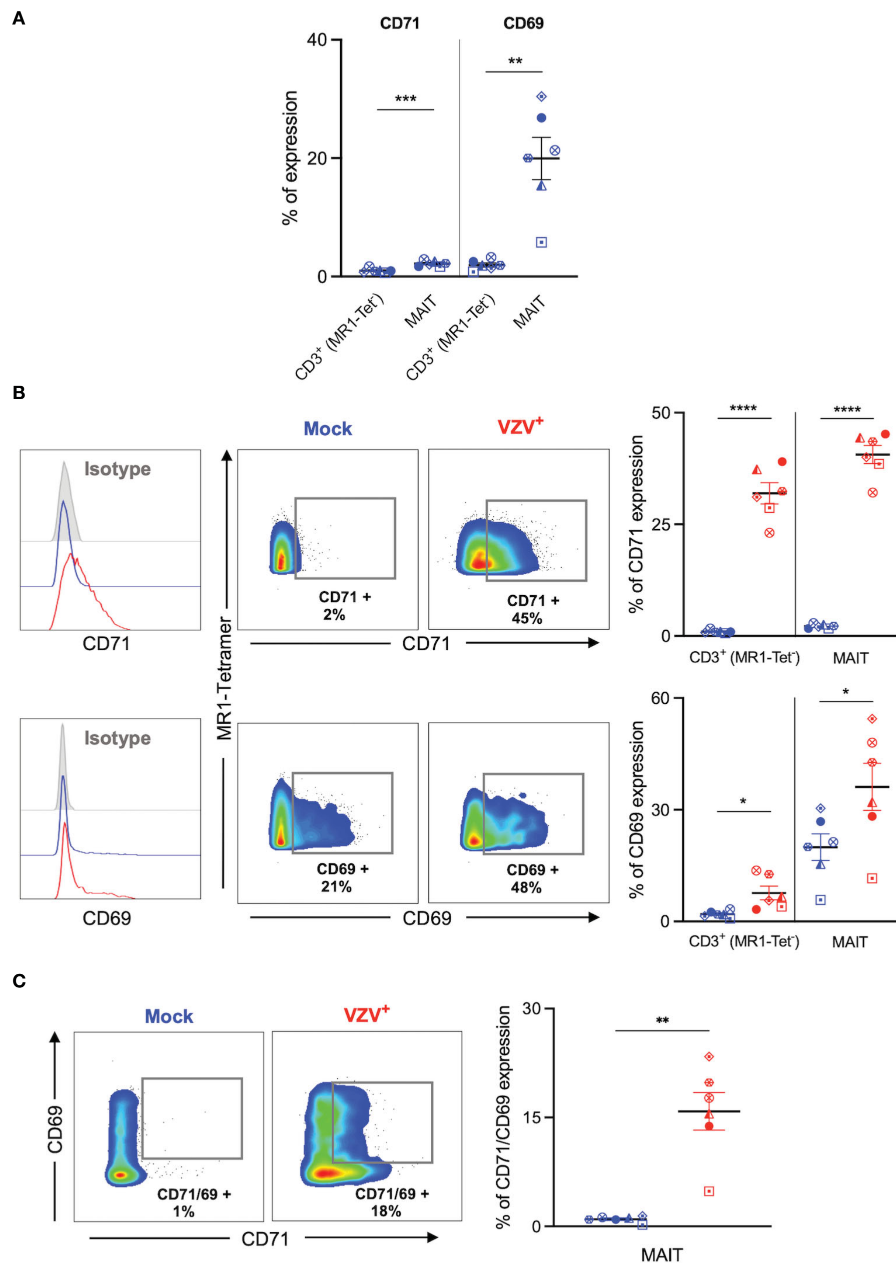

FIGURE 3
VZV infection of MAIT cells is associated with expression of early activation and proliferation markers. Human PBMCs were inoculated with mock or VZV-S infected ARPE-19 epithelial cells for 2 days and then analyzed for infection (gE:gI), proliferation (CD71), and early activation (CD69) markers by flow cytometry. **(A)** Graph shows comparative frequency of surface CD71 and CD69 expression in mock inoculated non-MAIT (ie MR1-Tet$^-$) CD3$^+$ T cells and MAIT cell populations, with symbols representing individual donors (n=6). Statistical analysis was performed *via* two tailed paired *t* test. **p<0.001, ***p<0.001. **(B)** Representative histograms show expression of CD71 and CD69 by MAIT cells for Mock (blue) and VZV infected (VZV$^+$) (red) populations corresponding to their respective isotype controls (filled grey). Flow cytometry plots show surface expression of CD71 and CD69 on MAIT cells for Mock (blue) and VZV infected (VZV$^+$) (red) populations. Graphs show frequency of CD71 and CD69 in non-MAIT (ie MR1-Tet$^-$) CD3$^+$ and MAIT cell subpopulations, with symbols representing individual donors, and mean and SEM indicated by the bars. Statistical analysis of CD71 and CD69 expression between Mock and VZV$^+$ infected non-MAIT CD3$^+$ cells and MAIT cells was performed *via* two tailed paired *t* test (n=6). *p<0.05, ****p<0.0001. **(C)** Flow cytometry plots show CD69 *vs* CD71 double expression on Mock (blue) and VZV infected (VZV$^+$) (red) populations. Graph shows frequency of CD69/CD71 double expressing MAIT cells, with symbols representing individual donors, and mean and SEM indicated by the bars. Statistical analysis of CD71 and CD69 expression between Mock and VZV$^+$ infected non-MAIT CD3$^+$ cells and MAIT cells was performed *via* two tailed paired *t* test (n=6). *p<0.05, ****p<0.0001.

possessing a potent program for extravasastion (9). In the context of VZV infection of MAIT cells, we observed no significant difference in proportion of infected cells expressing CCR2, CCR5 or CCR6 in comparison to mock infection (Figure 4B). The proportion of non-MAIT CD3$^+$ T cells expressing CCR2 and CCR6 were also not different between mock and VZV infection, whereas there was a significant increase in the proportion of non-MAIT CD3$^+$ T cells expressing CCR5 in VZV infection (mean 21.3%, SEM +/- 4.94%)

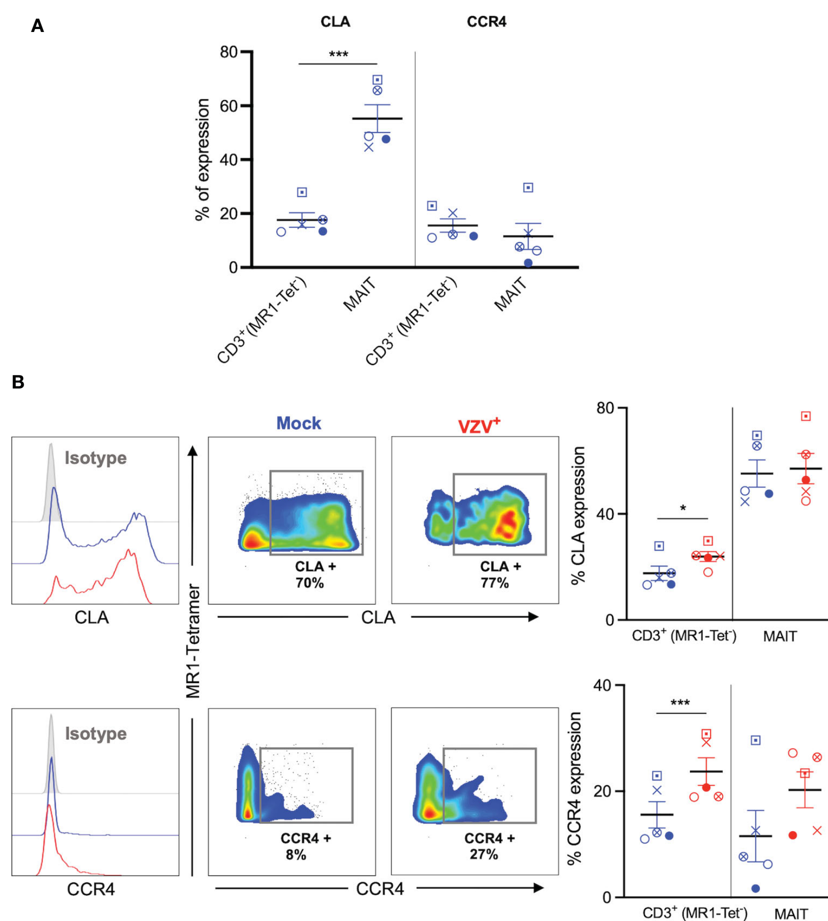

FIGURE 5
VZV infection of MAIT cells retains expression of CLA and CCR4 skin homing chemokine receptor expression. Human PBMCs were inoculated with mock or VZV-S infected ARPE-19 epithelial cells for 2 days and then analyzed for infection (gE:gI), and skin homing markers (CLA and CCR4) by flow cytometry. **(A)** Graph shows comparative frequency of surface CLA, and CCR4 expression in mock inoculated non-MAIT (ie MR1-Tet-) CD3+ T cells and MAIT cell populations, with symbols representing individual donors (n=5). Statistical analysis was performed *via* two tailed paired *t* test. ***p<0.001. **(B)** Representative histograms show expression of CLA and CCR4 by MAIT cells for Mock (blue) and VZV infected (VZV+) (red) populations corresponding to their respective isotype controls (filled grey). Flow cytometry plots from one donor show surface expression of CLA and CCR4 on MAIT cells for Mock (blue) and VZV infected (VZV+) (red) populations. Graphs show frequencies of non-MAIT CD3+ and MAIT cells expressing CLA and CCR4 with symbols representing individual donors across the subpopulations, with mean and SEM indicated by the bars. Statistical analysis of CLA and CCR4 expression between Mock and VZV+ infected non-MAIT CD3+ and MAIT cells was performed *via* two tailed paired *t* test (n=5). *p<0.05, ***p<0.001.

non-MAIT CD3+ cells demonstrated a significant upregulation of CLA compared to mock infected non-MAIT CD3+ cells (Figure 5B). Furthermore, there was a trend to an increased proportion of CCR4+ expression in VZV infected MAIT cells compared to mock (Figure 5B), whilst no change was observed in VZV bystander MAIT cells compared to mock (Supplementary Figure 4B). In addition, a greater proportion of CCR4+ non-MAIT CD3+ cells was observed during VZV infection compared to mock (Figure 5B). These data demonstrate that VZV infection of MAIT cells does not impair CLA or CCR4 expression and that VZV infection of non-MAIT CD3+ cells increases the expression of these skin homing markers.

Together, these results indicate that VZV infection of MAIT cells does not impair, but rather maintains expression of cell-surface cellular proteins associated with extravasation and skin homing programs.

MAIT cells support *de-novo* viral replication and virus transmission

Having established that MAIT cells were infected with VZV, we sought to determine whether VZV infected MAIT cells were capable transmitting infectious virus to other cells. We performed an infectious center assay which has been previously utilized to demonstrate productive infection and new infectious virion production in VZV infected T cells, NK cells and dendritic cells (29, 30, 35). MAIT cells were isolated by FACS sorting from PBMCs that had been exposed to a GFP-tagged VZV (VZV-ORF10-GFP) or had been mock infected (Figure 6A). The sorted MAIT cells were washed with citrate buffer to inactivate and detach any surface bound virions (41, 42, 49), before being co-cultured with uninfected ARPE-19 epithelial cell monolayers. After five days in culture, the

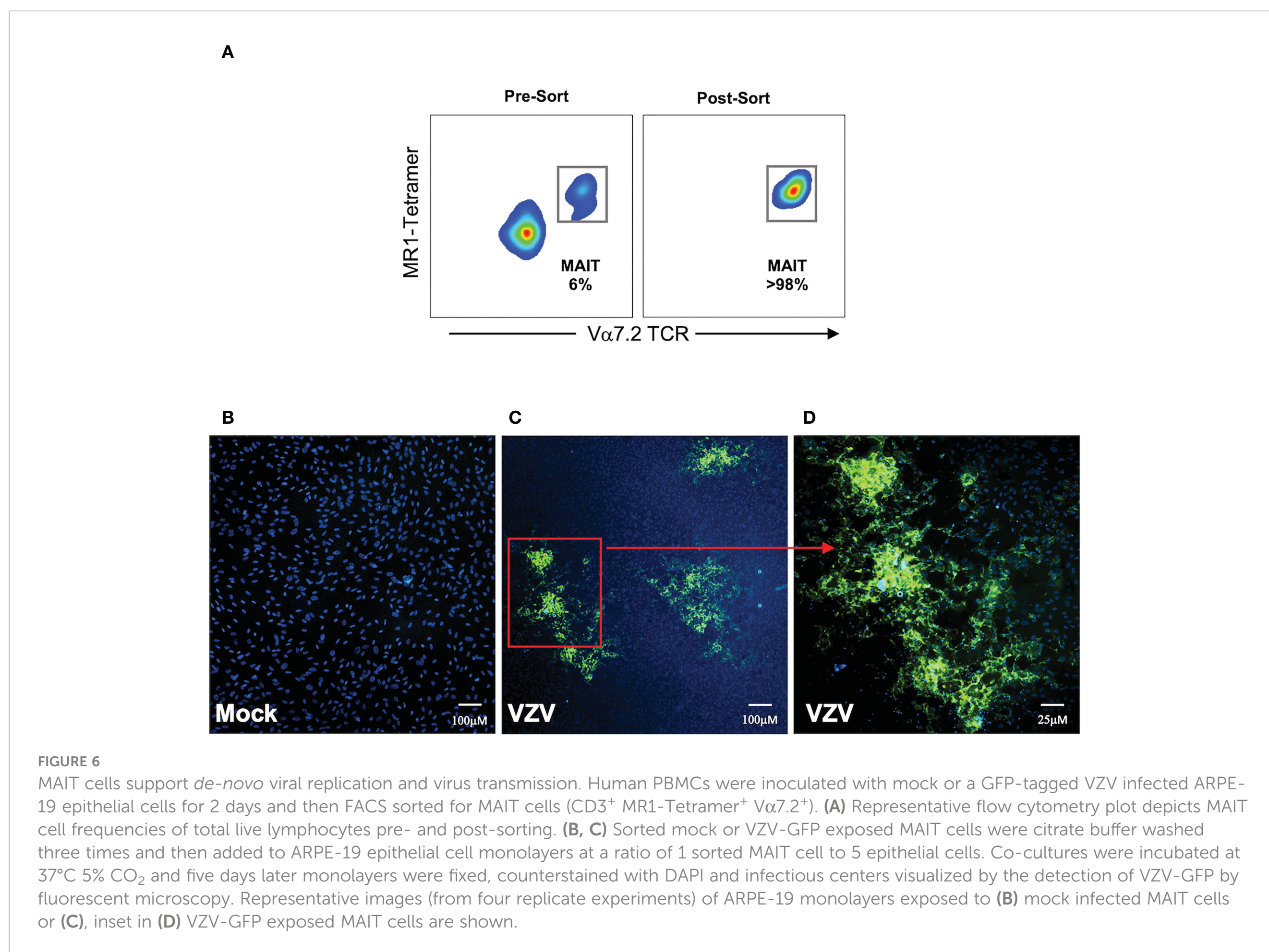

FIGURE 6
MAIT cells support *de-novo* viral replication and virus transmission. Human PBMCs were inoculated with mock or a GFP-tagged VZV infected ARPE-19 epithelial cells for 2 days and then FACS sorted for MAIT cells ($CD3^+$ $MR1\text{-}Tetramer^+$ $V\alpha7.2^+$). **(A)** Representative flow cytometry plot depicts MAIT cell frequencies of total live lymphocytes pre- and post-sorting. **(B, C)** Sorted mock or VZV-GFP exposed MAIT cells were citrate buffer washed three times and then added to ARPE-19 epithelial cell monolayers at a ratio of 1 sorted MAIT cell to 5 epithelial cells. Co-cultures were incubated at 37°C 5% CO_2 and five days later monolayers were fixed, counterstained with DAPI and infectious centers visualized by the detection of VZV-GFP by fluorescent microscopy. Representative images (from four replicate experiments) of ARPE-19 monolayers exposed to **(B)** mock infected MAIT cells or **(C)**, inset in **(D)** VZV-GFP exposed MAIT cells are shown.

presence of virus-induced cytopathic effect (CPE) in the ARPE-19 monolayer was determined *via* detection of GFP signal by fluorescence microscopy (Figures 6C, D). ARPE-19 cell monolayers co-cultured with Mock infected MAIT cells yielded no GFP fluorescence (Figure 6B) whereas distinct GFP^+ infectious centers were readily detected in ARPE-19 monolayers co-cultured with MAIT cells infected with VZV (Figures 6C, D). These data demonstrate that that human MAIT cells infected with VZV are capable of transmitting infectious virus to epithelial cells.

Discussion

A hallmark of VZV primary infection is the dissemination of cell-associated virus in infected individuals (27). Uncovering the full repertoire of immune cell populations that VZV infects is crucial in forming a better understanding of the key pathogen-host interactions that result in the widespread manifestation of cutaneous vesicles and subsequent inter-host transmission of virus (27, 50, 51). In this study, we provide evidence that VZV productively infects blood-derived MAIT cells, with a consequence being a capacity to transmit infectious virus to epithelial cells. We also demonstrate that VZV infected MAIT cells display a modulated activation status whilst retaining a highly expressed extravasation and skin homing program.

Cell-associated VZV infection of human PBMCs revealed infection of MAIT cells that was at a similar magnitude to non-MAIT $CD4^+$ and $CD8^+$ T cells. Furthermore, VZV infection did not alter the overall viability of MAIT cells. This is consistent with *in vitro* studies which reported no significant loss of viability of VZV infected T cells, NK cells or DCs (29–31, 35, 52), but contrasts with the rapid death of MAIT cells during *in vitro* infection by measles virus (26).

A recent study uncovered Siglec-7 (CD328) as a key cell receptor that binds to VZV entry glycoprotein (g)B and mediates the entry of VZV in monocytes (53, 54). However, Siglecs are poorly expressed in human T cells due to their potentially negative regulation of TCR signalling (55–57). Whilst the lack of Siglec-7 expression by T cells may potentially explain the higher level of infection observed in monocytes (45, 52–54), it also suggests a potentially distinct T lymphocyte specific entry receptor through which VZV gains entry.

Despite the extraordinary level of conservation present within the MR1-MAIT cell axis, there is a growing understanding of phenotypic and functional heterogeneity present within circulating MAIT cells. Indeed, MAIT cell expression of NK cell

associated markers such as CD56 is associated with a greater propensity for response to cytokine stimulation (46). We found no preference of VZV infection across MAIT cells either expressing or non-expressing CD56, CD27 or PD-1, nor did we observe frequency alterations of the subpopulations studied. Whilst we did demonstrate a significantly higher proportion of infection within $CD4^+$ and $CD4^+/CD8^+$ MAIT cells, the biological impact of this is not clear given they represent a small subset of the overall MAIT cell compartment (1).

Interestingly, analysis of the activation marker CD69, revealed that VZV infection was associated with a significantly higher proportion of $CD69^+$ MAIT cells compared to mock infection in both the context of whole PBMCs and MAIT cells FACS sorted on the basis of CD69 expression. These finding are similar to earlier studies that demonstrate an upregulation of CD69 in tonsillar T cells as a consequence of VZV infection (33, 34, 58, 59). This is similar with several lymphotropic viruses as they also require T cell activation for productive infection. Indeed, HIV-1 infection of resting naïve $CD4^+$ T cells results in an abortive non-replicative infection, whilst either mitogenic or anti-CD3/CD28 mediated stimulation of T cells drives production of replicating virus (60–62). Similarly, viral replication is increased when either VZV infected T cells or NK cells are treated with stimuli such as PMA or IL-2 respectively (30, 35). Like CD69, the proportion of MAIT cells expressing the T cell proliferation marker CD71 was significantly higher during VZV infection compared to mock infection, although we did not observe an overall difference in the number of MAIT cells between mock and VZV infected cultures. As CD71 plays a role in initiating proliferation and activation in resting, quiescent or terminally differentiated lymphocytes through permitting and accommodating for an increased metabolic demand (63–69), the upregulation of CD71 may rather reflect the metabolically higher demand of generating viral progeny.

Upregulation of skin homing markers on VZV infected T cells and NK cells has been reported (30, 33–35), yet there are no studies that observe markers of extravasation potential by VZV infected lymphocytes; a crucial step lymphocytes must take before accessing skin sites. In light of work by Lee et al., 2018 which elegantly demonstrated a highly expressed extravasation program by MAIT cells (9), we determined if VZV infected MAIT cells are likely to retain this potential. Similar to previous reports, MAIT cells endogenously expressed greater levels of CCR2, CCR5 and CCR6 compared to non-MAIT $CD3^+$ cells (9). Importantly, VZV infected MAIT cells were able to retain the constitutively high expression of CCR2, CCR5 and CCR6. Under homeostatic conditions, the skin homing capacity of circulating T cells is tightly regulated, whilst chronic skin conditions such as psoriasis are characterized by infiltrating T cells with markedly increased levels of CCR4 and CLA expression (70, 71). We found that VZV infected MAIT cells maintained a pronounced expression of CLA along with a trend to upregulated CCR4 expression. Studying the skin homing capacity of VZV infected MAIT cells *in vivo* is challenging given the high-species specificity of this virus and the lack of an animal model to study productive infection and MAIT cells (27). Migration assays utilizing a cognate skin homing chemokine such as CC chemokine ligand (CCL) 17 would illuminate whether VZV infected MAIT cells have a functional migration capacity, as shown by VZV infected tonsillar T cells (31).

The clinical manifestation of varicella, with widespread cutaneous lesions suggests a requirement for transfer of virus from the infected lymphocyte carrier to target keratinocytes (27, 72). The formation of distinct infectious centers in epithelial cell monolayers after incubation with VZV infected MAIT cells highlights a previously unknown target immune cell sub-population that may enable such infectious virus transfer from circulating cells to cutaneous sites. Whilst VZV is able to maintain and upregulate skin homing markers on NK cells, they remain functionally incapacitated to stimulation (35, 36). Whether VZV infection of MAIT cells represents a strategy for virus dissemination whilst also modulating MAIT cell effector functions such as cytokine production and cytolytic activity, and/or whether MAIT cells exert any VZV effector function remain important areas of investigation, particularly in the context of emerging evidence of a role for MAIT cells in a range of other virus infections (21–25).

Our understanding of the range of immune cell populations that VZV infects and manipulates to achieve inter-host dissemination is becoming increasingly diverse. Here we describe an immune cell subset permissive to VZV infection and propose MAIT cells to be a crucial target during VZV infection that contributes to the dissemination of virus.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by University of Sydney Human Research Ethics Committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

SP performed experiments. BS, AA and SP conceived the study and planned experiments. AC provided key reagents. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AC is an inventor on patents describing MR1-tetramers.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer LH declared a shared affiliation with the author AC to the handling editor at time of review.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1121714/full#supplementary-material

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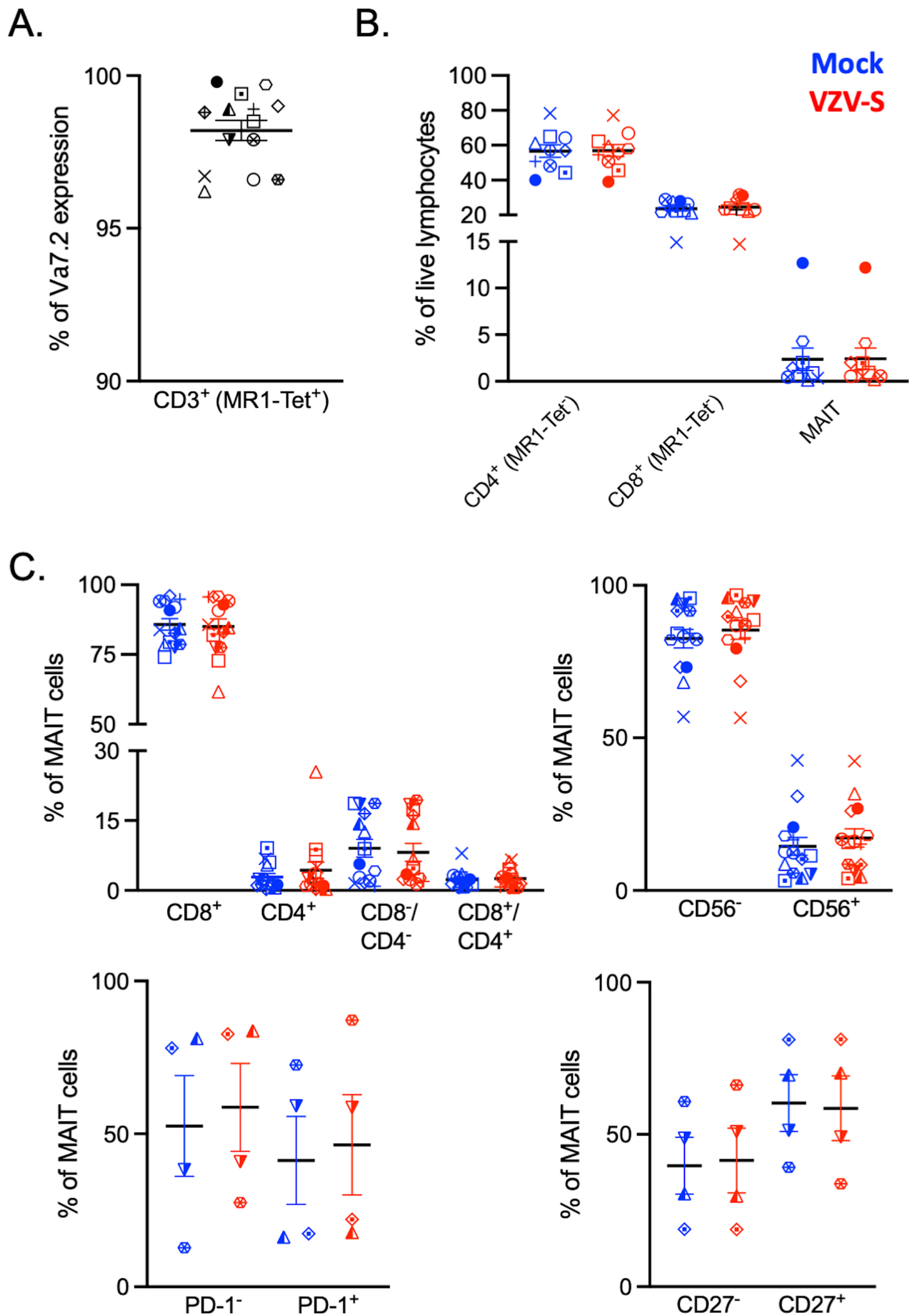
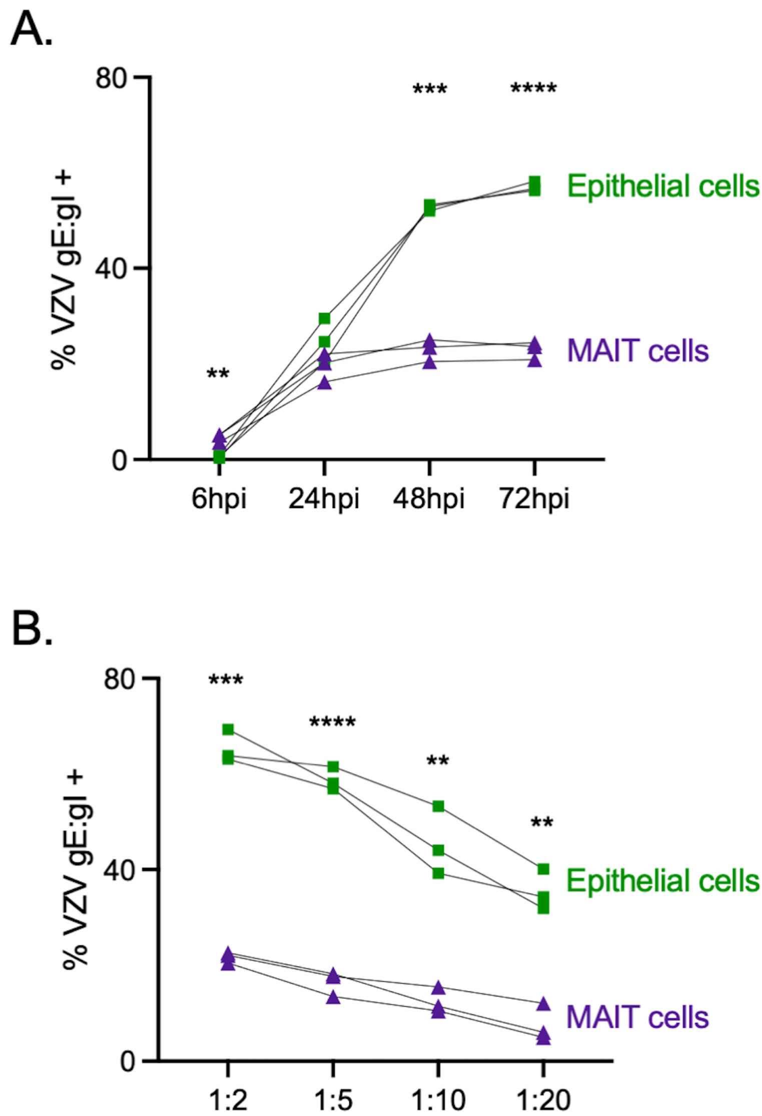


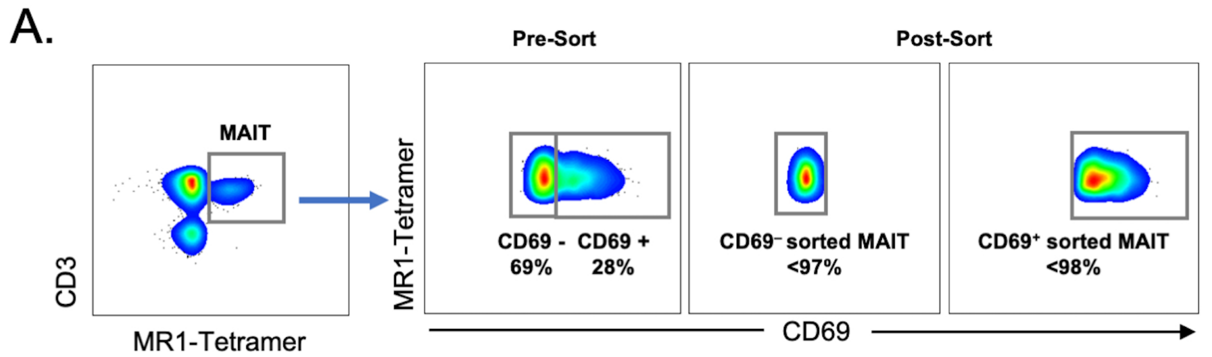
Supplementary Figure 1.

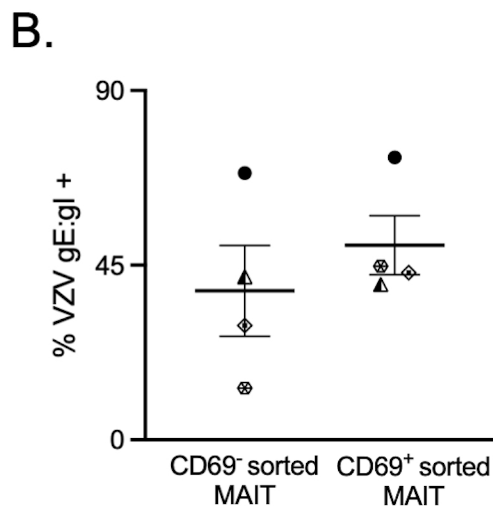
Human PBMCs were inoculated with mock or VZV-S infected ARPE-19 epithelial cells for 2 days. (A) Graph shows frequency of $CD3^+$ MR1-Tetramer$^+$ cells that positively co-stained for $V\alpha7.2^+$ (n=14). (B) Graphs shows frequencies of non-MAIT (ie MR1-Tet$^-$) $CD3^+$ $CD4^+$, non-MAIT (ie MR1-Tet$^-$) $CD3^+$ $CD8^+$ cells and MAIT cells as the percentage of total live lymphocytes for mock (blue) and VZV inoculated samples (red) (n=14). (C) Graphs show frequencies of MAIT cell co-receptor subpopulations: $CD8^+$, $CD4^+$, $CD8^-/CD4^-$, $CD8^+/CD4^+$ (n=14) as well as CD56 (n=14), PD-1 (n=4) and CD27 (n=4) expressing and non-expressing MAIT cells for mock (blue) and VZV inoculated (red) samples. Symbols represent individual donors across the subpopulations, with mean and SEM indicated by the bars. Statistical analysis of differences in lymphocyte frequencies between mock and VZV inoculated samples was performed via two tailed paired *t* test.

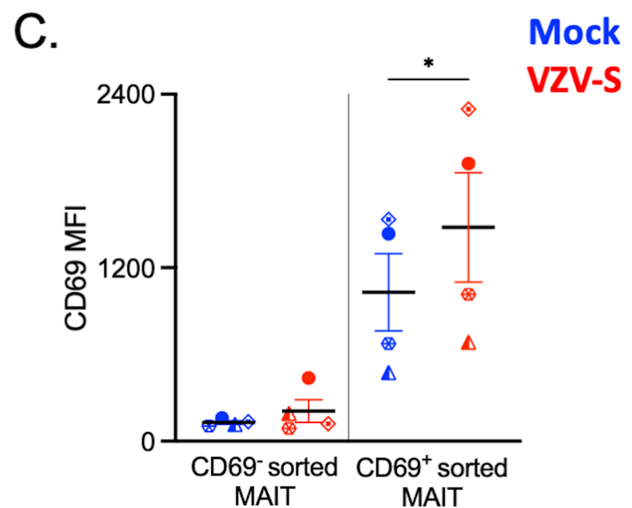


Supplementary Figure 2.

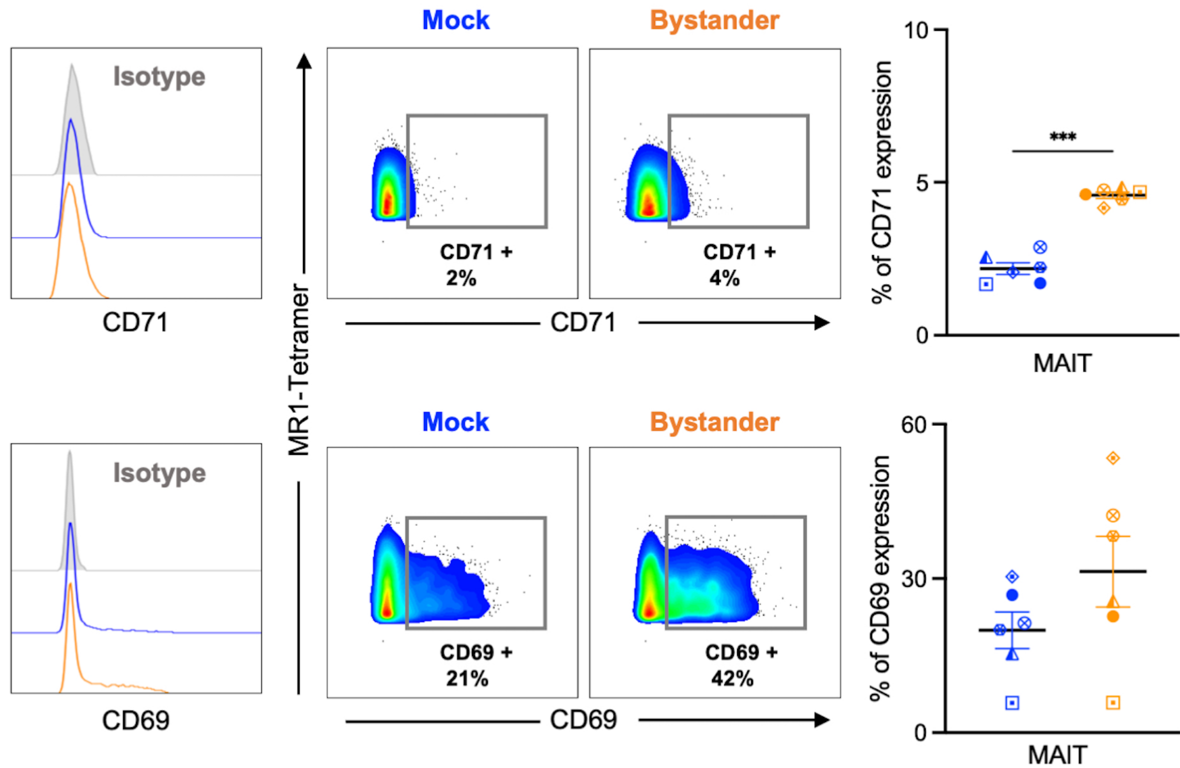
Human PBMCs or ARPE-19 GFP expressing cells (A-19-GFP) were inoculated with mock of VZV-S infected ARPE-19 epithelial cells at varying co-culture ratios as well as harvested at various time points post inoculation and analysed for infection (gE:gI) by flow cytometry. (A) Graph shows frequency of gE:gI expression at various time points post inoculation (1:5 inoculum: target) for MAIT cells (filled purple triangles) compared to A-19-GFP cells (filled green squares) at 6, 24, 48 and 72 hours post inoculation (n=3). (B) Graph shows frequency of gE:gI expression by MAIT cells (filled purple triangles) compared to A-19-GFP cells (filled green squares) after co-culturing with VZV-S inoculum for 48 hours at various inoculum: target ratios: 1:2, 1:5, 1:10, and 1:20 (n=3). Statistical analysis of gE:gI expression between MAIT and ARPE-19 GFP cells was performed via unpaired *t* test with Welch's correction (n=3). **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

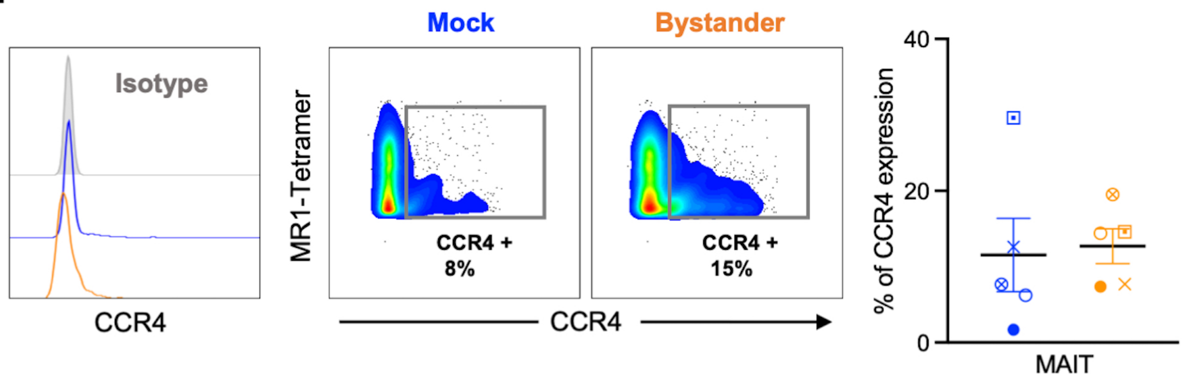






Supplementary Figure 3.
Human PBMCs were FACS sorted for CD69 expressing (CD69^{+}) and non-expressing (CD69^{-}) MAIT cells and inoculated with mock or VZV-S infected ARPE-19 epithelial cells for 2 days before analysing for infection (gE:gI) and early activation (CD69) expression. (A) Representative flow cytometry plot depicts CD69^{+} and CD69^{-} MAIT cell frequencies of total MAIT cells pre- and post-sorting. (B) Graph shows frequency of gE:gI expression for CD69^{+} and CD69^{-} sorted MAIT cells (n=4). Symbols represent individual donors across the sorted populations, with mean and SEM indicated by the bars. Statistical analysis of gE:gI expression between CD69^{+} and CD69^{-} sorted MAIT cells was performed via two tailed paired *t* test (n=4). (C) Graph shows the median fluorescence intensity (MFI) of CD69 expression on mock (blue) or VZV infected (red) CD69^{+} and CD69^{-} sorted MAIT cells (n=4). Symbols represent individual donors across the sorted populations, with mean and SEM indicated by the bars. Statistical analysis of CD69 MFI between mock and VZV infected CD69^{+} and CD69^{-} sorted MAIT cells was performed via two tailed paired *t* test (n=4). *$p<0.05$.





Supplementary Figure 4.
Human PBMCs were inoculated with mock or VZV-S infected ARPE-19 epithelial cells for 2 days. (A) Representative histograms show expression of CD71 and CD69 by MAIT cells for Mock (blue) and VZV bystander (gE:gI-) (yellow) populations corresponding to their respective isotype controls (filled grey). Flow cytometry plots show surface expression of CD71 and CD69 on MAIT cells for Mock (blue) and VZV bystander (gE:gI-) (yellow) populations. Graphs show frequency of CD71 and CD69 in Mock (blue) and VZV bystander (gE:gI-) (yellow) MAIT cell populations with symbols representing individual donors, and mean and SEM indicated by the bars. Statistical analysis of CD71 and CD69 expression between Mock and VZV bystander MAIT cells was performed via two tailed paired *t* test (n=6). ***$p<0.001$. (B) Representative histograms show expression of CCR4 by MAIT cells for Mock (blue) and VZV bystander (gE:gI-) (yellow) populations corresponding to their respective isotype controls (filled grey). Flow cytometry plots show surface expression of CCR4 on MAIT cells for Mock (blue) and VZV bystander (gE:gI-) (yellow) populations. Graphs show frequency of CCR4 in Mock (blue) and VZV bystander (gE:gI-) (yellow) MAIT cell populations with symbols representing individual donors, and mean and SEM indicated by the bars. Statistical analysis of CCR4 expression between Mock and VZV bystander MAIT cells was performed via two tailed paired *t* test (n=5).

Chapter 4. Functional impairment of Mucosal Associated Invariant T cells by Varicella Zoster Virus

4.1 Introductory statement

The findings of Chapter 3 identified MAIT cells as permissive to VZV infection, and furthermore demonstrated the ability of MAIT cells to transmit infectious virus to epithelial cells. However, transmission and infection *in vivo* triggers several pro-inflammatory responses (as reviewed in Chapter 1.2); potentially activating both the skin resident and recruited MAIT cells. Therefore, we hypothesised that VZV counters this potential immune cell activation by disrupting MAIT cell functionality. Thus, we investigated the capacity for MAIT cells to functionally respond to diverse stimulation conditions following VZV inoculation.

The findings generated from this study was published in PLOS Pathogens in 2024 and forms Chapter 4 of this thesis. The materials and methods used for generating the data within this publication are all enclosed as a subsection within the paper and not a separate thesis chapter. Similarly, all literature citations and figures associated with supplementary information are included as subsections within the paper.

PLOS PATHOGENS

RESEARCH ARTICLE

Varicella Zoster Virus disrupts MAIT cell polyfunctional effector responses

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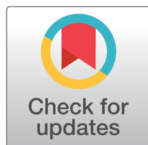

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Competing interests: J.Y.W.M., A.J.C., and D.P.F. are inventors on patents describing MAIT cell

Abstract

Mucosal-associated invariant T (MAIT) cells are unconventional T cells that respond to riboflavin biosynthesis and cytokines through TCR-dependent and -independent pathways, respectively. MAIT cell activation plays an immunoprotective role against several pathogens, however the functional capacity of MAIT cells following direct infection or exposure to infectious agents remains poorly defined. We investigated the impact of Varicella Zoster Virus (VZV) on blood-derived MAIT cells and report virus-mediated impairment of activation, cytokine production, and altered transcription factor expression by VZV infected (antigen+) and VZV exposed (antigen-) MAIT cells in response to TCR-dependent and -independent stimulation. Furthermore, we reveal that suppression of VZV exposed (antigen-) MAIT cells is not mediated by a soluble factor from neighbouring VZV infected (antigen+) MAIT cells. Finally, we demonstrate that VZV impairs the cytolytic potential of MAIT cells in response to riboflavin synthesising bacteria. In summary, we report a virus-mediated immune-evasion strategy that disarms MAIT cell responses.

Author summary

Mucosal-associated invariant T (MAIT) cells are a uniquely specialised and substantial innate-T cell population that can rapidly respond to diverse bacterial and fungal pathogens through T cell receptor dependent recognition of riboflavin synthesis derived metabolite antigens. Additionally, MAIT cells can be triggered by local pro-inflammatory cues such as cytokines; therefore extending their functionality to non-riboflavin pathogens such as viral infections. Despite the capacity of MAIT cells to play a protective role against several classes of pathogens, there remains a dearth of studies investigating direct pathogenic suppression of MAIT cell functionality. Here, we investigate a previously uncharacterised interplay between MAIT cells and the causative agent of varicella (chickenpox) and shingles (zoster): Varicella Zoster Virus (VZV). VZV successfully infects and establishes lifelong latency within the host; in part to their ability to effectively manipulate

antigens and tetramers. The authors declare no other potential conflict of interest.

several innate and adaptive axes of the host immune response. In this study, we report that VZV profoundly impairs MAIT cell activation in response to both riboflavin synthesis and cytokine stimulation, therefore resulting in a downstream paralysis of several effector functions such as cytokine production and cytotoxic potential. This work highlights a previously uncharacterised strategy of viral pathogens to effectively target and restrict the MAIT cell effector response.

Introduction

Varicella Zoster Virus (VZV) is a highly successful human pathogen that causes varicella during primary infection and can later reactivate from latency and result in herpes zoster (HZ). A defining characteristic of VZV pathogenesis is the virus' ability to encode several immune evasion strategies that impair pathogen detection systems whilst also infecting and disabling host effector immune cell populations. In particular, VZV evades classical T cell and Natural killer (NK) cell responses by downregulating surface expression of Major Histocompatibility Complex class-I [1], class II (MHC-II) [2], and NK cell activating ligands [3]. Furthermore, VZV productively infects and functionally disrupts several immune cell subsets such as monocytes [4], dendritic cells (DCs) [5,6], conventional T cells [7,8], and NK cells [9,10] enabling host wide hematogenous dissemination. A common feature of varicella infection is viremia accompanied with high viral load at respiratory mucosa and skin sites [11,12]. Clinically, primary varicella infection presents as cutaneous vesicular lesions which disrupt the normal skin architecture at these sites, and permit translocation of commensal microbes. Unsurprisingly, bacterial superinfections ranging from bacterial cellulitis to pneumonia and/or sepsis are a common complication arising from severe VZV infection [13–15].

Importantly, these barrier locations are also enriched with resident Mucosal Associated Invariant T (MAIT) cells [16,17] which typically express a semi-invariant T cell receptor (TCR) [18]. MAIT cells are the largest innate-adaptive immune cell population within the body and are exquisitely tuned to rapidly respond to deeply conserved microbial metabolic patterns expressed by commensal and pathogenic species. Specifically, the MAIT TCR binds to unstable microbial metabolite antigens such as 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU) derived from the Vitamin B2 (riboflavin) biosynthesis pathway presented by the MHC-I related molecule MR1 [19,20]. Riboflavin biosynthesis is a metabolic process that is broadly conserved across diverse bacterial and fungal species [21]; however is not present within viral or mammalian systems.

TCR dependent stimulation of MAIT cells drives co-expression of transcription factors: RAR-related orphan receptor γ T (RORγt) and T box 21 (T-bet) [22], leading to the expression of several cytokines such as Interferon (IFN)-γ, Tumour necrosis factor (TNF), interleukin (IL)-17 and IL-22, as well as cytolytic capacity denoted by granzyme B and perforin expression [22–24]. Consequently, the cognate MAIT TCR-MR1 interaction enables MAIT cells to enact rapid polyfunctional responses against both commensal and pathogenic riboflavin synthesising organisms at several key mucosal barrier sites [25–27].

Under homeostatic conditions, the constant and steady diffusion of riboflavin metabolites from commensals across mucosal interfaces drives a T cell effector type-17 (Tc-17) like barrier maintenance and tissue repair signature [17,28–30]. However, during an infection setting, an intact riboflavin synthesising pathogen would also trigger several toll-like receptors (TLRs) within the antigen presenting cell (APC), resulting in a concomitant delivery of MR1 antigen presentation and innate signals to MAIT cells [16,31]. Indeed, MAIT cells are highly

responsive to interleukin IL-18 and IL-12 signalling, which in combination with TCR stimulation drives a robust and prolonged functional response as well as proliferation [32].

Importantly, MAIT cells can be activated by cytokines such as IL-12 and IL-18 in the absence of TCR stimulation [33], extending the influence of MAIT cell functionality to viral and autoimmune diseases. Cytokine driven activation of MAIT cells drives a predominantly T-bet mediated response characterised by IFN-γ and granzyme B expression [22]. This robust anti-viral phenotype can result in protective efficacy against several viral infections such as Human Immunodeficiency Virus (HIV) [34], Hepatitis B Virus (HBV) [35], and Influenza [36].

Despite the ability of MAIT cells to rapidly respond to, and coordinate immune responses to, a vast array of pathogens, pathogen encoded strategies that directly impair MAIT cell effector functionality remain understudied. Our previous studies have revealed both modulation of MR1 antigen presentation [37], and direct infection of MAIT cells [38], therefore suggesting a direct targeting of the host MR1-MAIT cell axis by VZV for pathogenic gain. Therefore, this current study aimed to interrogate the direct impacts on MAIT cell functionality following VZV infection. Herein, we demonstrate a profound inhibition of MAIT cell response to both TCR-dependent and -independent activation, as shown by a significant abrogation of activation and cytokine expression. Strikingly, the suppression of effector response was observed in both VZV antigen positive as well as antigen negative MAIT cells. Finally, we reveal an impairment of cytotoxic capacity towards intact bacteria in VZV co-cultured MAIT cells. Overall, our report demonstrates a direct impact of viral infection on MAIT cell effector functionality following viral interaction and infection.

Results

VZV impairs MAIT cell activation

We sought to assess the direct outcome of VZV infection on MAIT cell functionality. Using a cell-associated infection model that closely replicates *in vivo* transmission of virus [38], we co-cultured VZV infected epithelial cells with human peripheral blood mononuclear cells (PBMCs). Following 24 hours of co-culture, we assessed MAIT cell responses to four distinct treatment conditions: 1. 5-OP-RU (TCR ligand treatment), 2. IL-12/IL-18 (cytokine treatment), 3. 5-OP-RU + IL-12/IL-18 (combination treatment) and 4. DMSO control (untreated condition).

MAIT cells were identified via co-staining of 5-OP-RU loaded MR1 tetramer and CD3 (Fig 1A). Consistent with previous literature, we detected MAIT cell numbers at an average of 2.2% of live T cells; with no difference in frequency observed between mock and VZV co-cultured MAIT cells (S1A Fig). A widely utilised readout of VZV infection being the detection of surface viral glycoprotein E (gE):gI complex (expressed late in VZV replication cycle) [4,8–10,38,39] was employed to classify MAIT cells as either virally infected or exposed (Fig 1A). We observed a significant increase in the percentage of gE:gI expression in TCR ligand (5-OP-RU) treated MAIT cells (mean 22.5%) compared to untreated MAIT cells (mean 9.8%) (S1B Fig), suggesting that TCR stimulation may promote a greater infection of MAIT cells.

Using surface CD69 and PD-1 expression as markers of early activation, we observed a robust expression of CD69 and PD-1 in mock co-cultured MAIT cells for TCR ligand, cytokine and combination treatment groups (Fig 1B and 1C). Comparatively, a significant reduction of both CD69 and PD-1 expression was demonstrated by VZV infected MAIT cells across all treatment groups (Fig 1C). Interestingly, CD69 expression of VZV exposed and infected MAIT cells was significantly lower compared to mock in the untreated condition (Fig 1C), suggesting that VZV may alter the underlying response potential of MAIT cells.

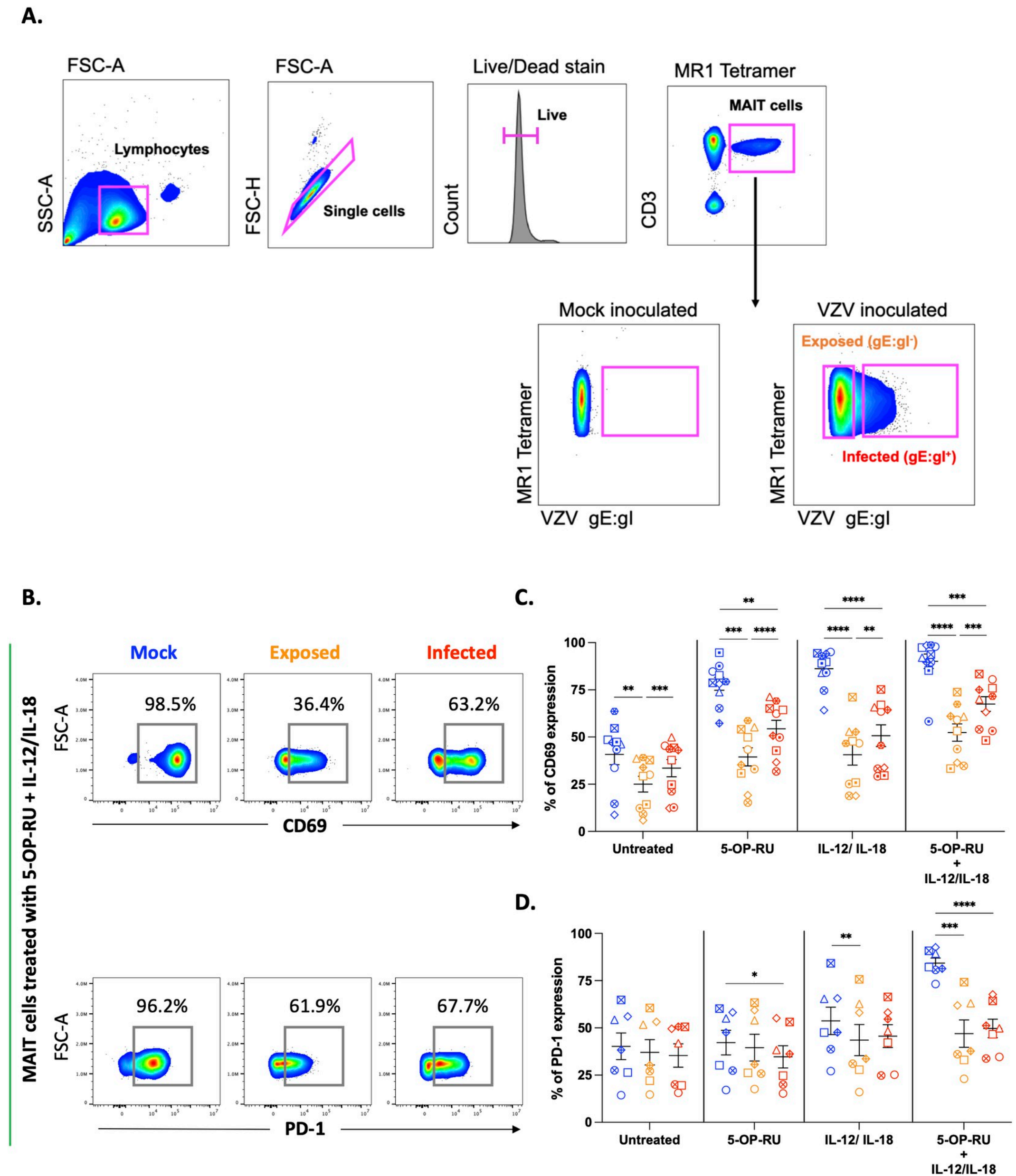

Fig 1. VZV impairs MAIT cell activation. Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for one day. PBMCs were removed from co-culture, before treating with different stimulations as specified, and then analysed by flow cytometry. **(A)** Representative flow cytometry plots depict the gating strategy to identify MAIT cells in PBMCs as $CD3^+$ and MR1-$Tetramer^+$. Following MAIT cell identification, surface VZV glycoprotein (g)E:gI complex staining was used to subgroup MAIT cells as either Infected ($gE{:}gI^+$) or Exposed ($gE{:}gI^-$). **(B)** Flow cytometry plots depict surface expression of CD69 and PD-1 of mock, exposed and infected MAIT cells in response to 5-OP-RU + IL-12/IL-18 treatment. **(C and D)** Graphs show frequency of CD69 **(C)** and PD-1 **(D)** expression of mock (blue), exposed (orange) and infected (red) MAIT cells to treatments as specified with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing CD69 **(C)** and PD-1 **(D)** expression between mock, exposed and infected MAIT cells within each treatment group was performed via Two-Way repeated measures ANOVA (n = 10). $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

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Strikingly, VZV exposed MAIT cells exhibited significantly lower activation levels compared to both mock and VZV infected MAIT cells across all treatment groups (Fig 1C). To examine any relationship between the magnitude of MAIT cell infection and activation status:

we performed correlation analysis between the fluorescence intensity of gE:gI staining and CD69 expression and found no correlation.

Viral infection of MAIT cells, can result in apoptotic programming and death; as seen with measles virus infection [40]. Therefore, we examined MAIT cell apoptosis levels following VZV inoculation by utilising a well-established flow cytometric based detection of intracellular cleaved Caspase-3 as a readout for apoptosis [41]. We found that VZV exposed and infected MAIT cells did not exhibit significantly greater levels of apoptosis compared to mock (S2A and S2B Fig). Therefore indicating the observed impairment of MAIT cell activation by VZV to not be likely a result of apoptotic programming.

Taken together, these results suggest VZV drives an impairment of MAIT cell activation in both the directly infected ($gE:gI^{+}$) as well as exposed ($gE:gI^{-}$) cells. Furthermore, this impairment was consistently observed in TCR-dependent, cytokine driven and combination treatment groups, suggesting a potentially global paralysis of MAIT cell response to several distinct modalities of stimulation.

VZV co-cultured MAIT cells are functionally refractory to both TCR-dependent and cytokine driven stimulation

Next, we sought to determine whether the lack of activation in VZV co-cultured MAIT cells corresponds to a reduction of cytokine and granzyme expression. Intracellular expression of granzyme B, IFN-γ, and TNF was assessed by flow cytometry to evaluate MAIT cell pro-inflammatory functional responses. In mock, VZV exposed and VZV infected MAIT cells, minimal granzyme B, IFN-γ and TNF expression was observed in the untreated condition (Fig 2). Stimulation of mock MAIT cells with combination treatment resulted in the most notable increase of granzyme B expression (83-fold increase compared to untreated condition) (Fig 2B and 2E). Increase of granzyme B expression was also observed for VZV infected MAIT cells across all treatment conditions compared to the untreated condition (Fig 2E). However, compared to mock, VZV infected MAIT cells demonstrated a significantly reduced ability to express granzyme B across all modalities of activation (Fig 2B). Expression of IFN-γ was the highest in combination treated mock MAIT cells (61-fold increase compared to untreated condition), which was also observed albeit to a lesser extent in VZV infected MAIT cells (5-fold increase compared to untreated condition). Across all stimulation conditions, VZV infected MAIT cells exhibited a significant inhibition of IFN-γ expression (Fig 2C). Interestingly, TCR ligand treatment of mock MAIT cells induced the greatest level of TNF expression (65-fold increase compared to untreated condition), whilst cytokine treatment of MAIT cells failed to significantly induce TNF expression as previously observed [22] (Fig 2D and 2G). Again, VZV infected MAIT cells failed to significantly express TNF in any stimulation groups when compared to mock (Fig 2D). Interestingly, a minor but significant upregulation of TNF was observed in the untreated condition by VZV infected MAIT cells (mean 1.1%) compared to mock (mean 0.17%) (Fig 2D).

Strikingly, VZV exposed MAIT cells demonstrated the greatest impairment in expressing granzyme B, IFN-γ, and TNF compared to mock across TCR dependent and TCR independent forms of stimulation (Fig 2). To further characterise whether the VZV exposed MAIT cell subpopulation was *bona-fide* exposed or in early infection stages, we infected PBMCs with VZV inoculum for 24 hours before FACS sorting on gE:gI negative MAIT cells and then culturing for 24 and 48 hours in isolation (S3A Fig). We observed that almost all gE:gI negative MAIT cells remained gE:gI negative at both 24 and 48 hours suggesting these cells to be likely resistant to productive infection (S3B Fig).

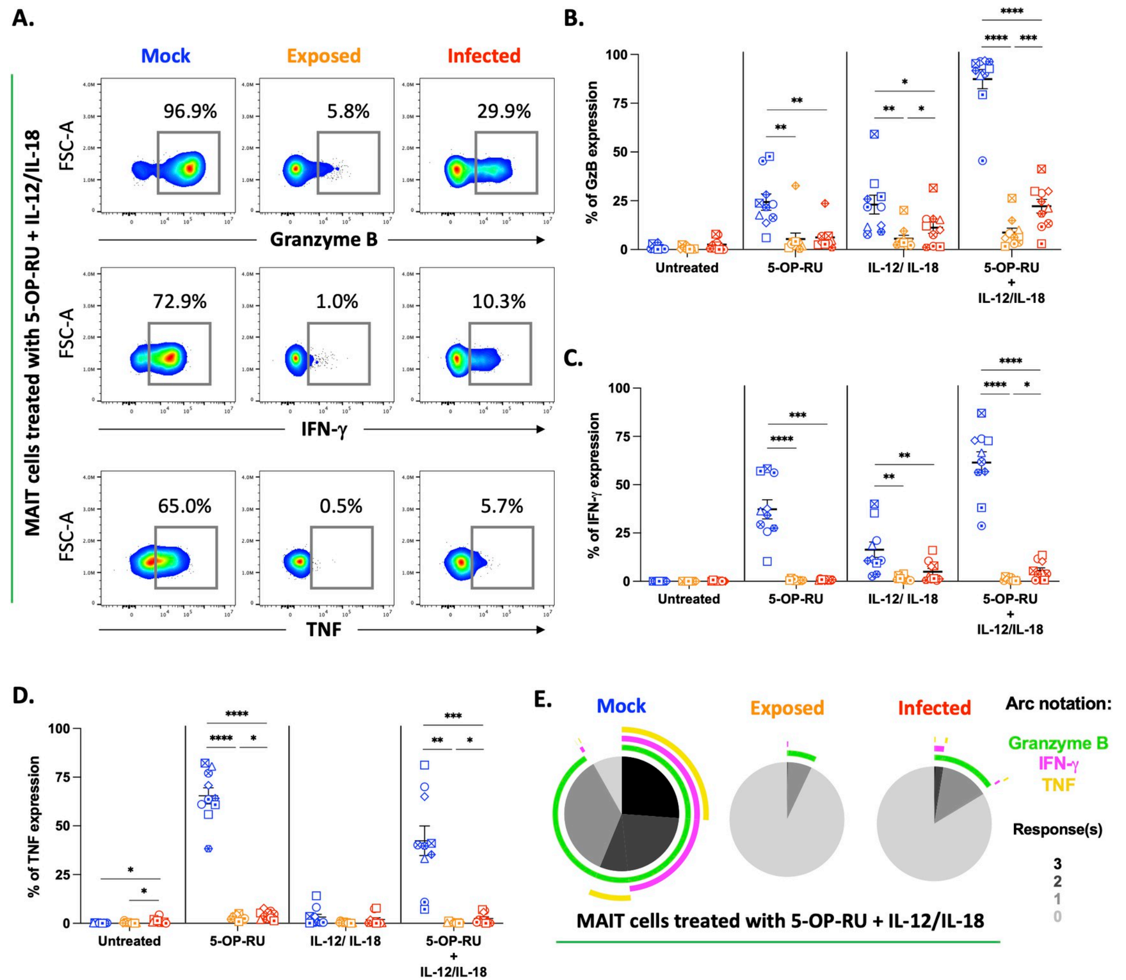

Fig 2. VZV co-cultured MAIT cells are functionally refractory to both TCR dependent and cytokine driven stimulation. Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for one day. PBMCs were removed from co-culture, before treating with different stimulations as specified, and then analysed by flow cytometry. **(A)** Flow cytometry plots depict intracellular expression of granzyme B, IFN-γ and TNF of mock, exposed and infected MAIT cells in response to 5-OP-RU + IL-12/IL-18 treatment. **(B,C and D)** Graphs show frequency of granzyme B **(B)**, IFN-γ **(C)** and TNF **(D)** expression of mock (blue), exposed (orange) and infected (red) MAIT cells to treatments as specified with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing Granzyme B **(B)**, IFN-γ **(C)** and TNF **(D)** expression between mock, exposed and infected MAIT cells within each treatment group was performed via Two-Way repeated measures ANOVA (n = 10). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **(E)** SPICE pie charts show the proportion of responses by mock, exposed and infected MAIT cells to 5-OP-RU + IL-12/IL-18 stimulation, based on the combinations of granzyme B, IFN-γ and TNF expression. Pie slices indicate the number of responses (0–3) (key, bottom right). Arcs depict the markers detected for each response (key, top right). SPICE data represents the mean of five donors.

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Next, we wanted to investigate whether suppression of MAIT cell cytokine expression may extend to lower infectious doses. Therefore, we inoculated PBMCs with a range of viral inoculum: PBMC ratios (1:5, 1:10, and 1:20). In line with our previous study: we observed a ratio-dependent infection of MAIT cells, with the 1:20 dose generating a significantly lower level of infection compared to the 1:5 dose [38], and this was consistent across all MAIT cell stimulation conditions (S4A Fig). Furthermore, we found that even at the lowest ratio of 1:20, both exposed and infected MAIT cells demonstrated significantly lower levels of CD69 and IFN-γ co-expression compared to mock infection across all stimulation conditions (S4B and S4C Fig).

Detection of IFN-γ and TNF co-expression in mock MAIT cells was only seen in the TCR ligand (mean 31.7%) and combination (ligand plus cytokines) treatment groups (mean 37.7%) (S5A and S5B Fig). In contrast, both VZV infected and exposed MAIT cells demonstrated almost a complete absence of IFN-γ and TNF co-expression for both ligand TCR and combination treatment groups (S5B Fig).

Furthermore, through Boolean gating of functional marker co-expression we utilised SPICE (Simplified Presentation of Incredibly Complex Evaluations) analysis to quantitatively describe the lack of polyfunctional response observed in bystander and infected MAIT cells compared to mock. SPICE analysis revealed that an average of 6% of TCR ligand stimulated mock MAIT cells co-expressed granzyme B, IFN-γ, and TNF (S5C Fig), whilst 26.4% of combination stimulated mock MAIT cells expressed all three functional markers (Fig 2E). This polyfunctional response to TCR as well as combination stimulation was notably absent in both VZV exposed and infected MAIT cells (Fig 2E). Collectively, we observed a profound inability for both VZV exposed and infected MAIT cells to express several functional markers in response to TCR dependent, TCR-independent or combined stimulation.

Differential expression of transcription factors in unstimulated and stimulated VZV co-cultured MAIT cells

The observation that VZV exposed and infected MAIT cells failed to *denovo* express key cytokine and cytolytic markers in response to various stimuli, raised the possibility that VZV potentially targets upstream regulators of protein synthesis such as transcription factors. Thus we assessed the expression of transcription factors T-bet and RORγt which serve as master regulators of the Th1 and Th17 response respectively. Interestingly, in the unstimulated condition we observed significantly increased mean fluorescence intensity (MFI) of T-bet and RORγt in VZV infected MAIT cells compared to mock (Fig 3A, 3B and 3C). Furthermore, an upregulation of T-bet MFI was observed in ligand TCR stimulated VZV infected MAIT cells (Fig 4B), whilst increased RORγt MFI was also observed in cytokine stimulated VZV infected MAIT cells (Fig 3C). In accordance with the activation and cytokine expression data, combination stimulation induced the greatest expression of T-bet and RORγt in mock MAIT cells (Fig 3B and 3C). Again, both VZV exposed and infected MAIT cells failed to upregulate T-bet and RORγt expression and demonstrated significantly lower MFI compared to combination stimulated mock MAIT cells (Fig 3B and 3C). Interestingly, VZV exposed MAIT cells exhibited lower T-bet and RORγt expression compared to VZV infected MAIT cells across all treatment groups (Fig 3B and 3C). This corresponds with earlier data (Figs 1 and 2) which also demonstrated consistently lower activation, cytokine and granzyme production by VZV exposed MAIT cells compared to those infected. The failure of VZV exposed and infected MAIT cells to upregulate T-bet and RORγt after combination stimulation correlates with the lack of cytokine and granzyme expression in response to stimulation. However, the lack of a complete abrogation of transcription factor expression suggests that VZV targets additional mechanisms in the T cell activation pathway to regulate MAIT cell functional response.

VZV impairment of MAIT cells is contact dependent and not mediated by soluble factors

The remarkable extent to which VZV exposed MAIT cells were functionally unresponsive to stimulation prompted the possibility that the inhibition of MAIT cells may be mediated by soluble factors within the viral co-culture supernatant. To test this, PBMCs were separated from mock or VZV inoculum by a transwell membrane and then stimulated through TCR dependent and independent modalities as previously described. The absence of gE:gI staining in

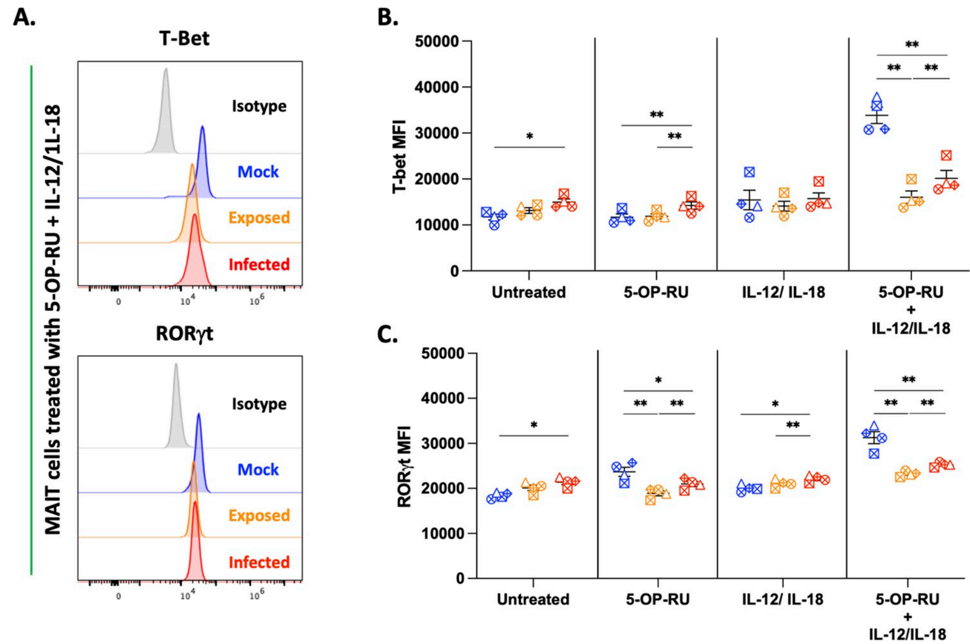

Fig 3. Differential expression of transcription factors in unstimulated and stimulated VZV and mock co-cultured MAIT cells. Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for one day. PBMCs were removed from co-culture, before treating with different stimulations as specified, and then analysed by flow cytometry. **(A)** Histograms depict intra-nuclear expression of T-bet and RORγt in mock, exposed and infected MAIT cells in response to 5-OP-RU + IL-12/IL-18 treatment. **(B and C)** Graphs show mean fluorescence intensity (MFI) of T-bet **(B)** and RORγt **(C)** of mock (blue), exposed (orange) and infected (red) MAIT cells to treatments as specified with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing T-bet **(B)** and RORγt **(C)** MFI between mock, exposed and infected MAIT cells within each treatment group was performed via Two-Way repeated measures ANOVA (n = 4). *$p<0.05$, **$p<0.01$.

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PBMCs demonstrated a lack of infection by VZV (Fig 4A). This was not surprising as VZV is highly cell-associated *in vitro* with extremely limited release of cell-free virions, therefore requiring cell-cell contact for transmission of virus [42]. Furthermore, we observed no difference in CD69 expression between mock and VZV co-cultured MAIT cells across all treatment conditions (Fig 4B and 4C), suggesting that cell-contact with viral inoculum is required for inhibition of MAIT cell response. However, this system does not exclude the possibility of an inhibitory secreted factor expressed after contact between viral inoculum and PBMCs. We therefore collected supernatant generated after 1 day of direct cell contact between mock or viral inoculum with PBMCs and incubated fresh PBMCs with mock or viral supernatant for 24 hours. As expected, incubation with viral supernatant did not result in infection of PBMCs (Fig 4D). Interestingly, viral supernatant induced greater expression of CD69 expression by MAIT cells in the untreated, ligand TCR and cytokine stimulated conditions compared to mock supernatant (Fig 4E and 4F). Taken together, the data suggest that impairment of MAIT cell response to stimulation by VZV is contact dependent and not mediated by soluble factors.

VZV impairs cytolytic potential of MAIT cells towards bacterially treated cells

Next, we wanted to determine whether VZV impairment of MAIT cell functions extended to a more physiologically reflective model of MAIT cell response to intact bacteria. We examined the expression of CD107a, granzyme B and perforin to evaluate the cytolytic potential of VZV co-cultured MAIT cells in response to intact bacteria. Briefly, THP-1 cells (antigen presenting

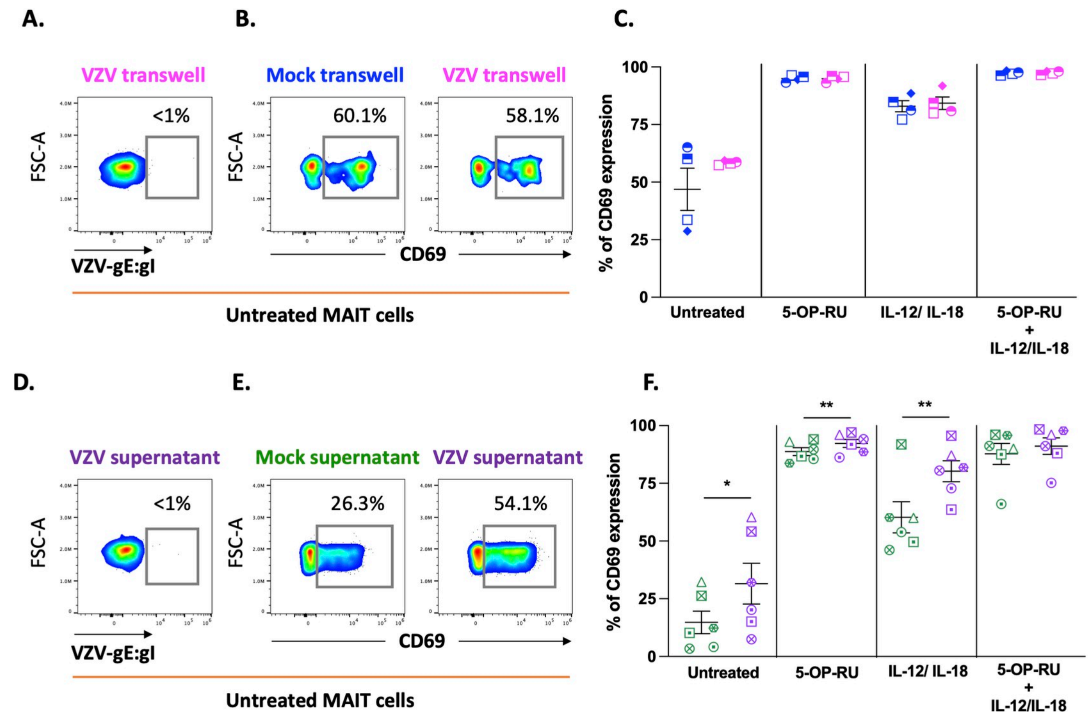

Fig 4. VZV impairment of MAIT cells is contact dependent and not mediated through soluble factors. (A, B and C) Human PBMCs were separated using a transwell system from mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells and co-incubated for one day. PBMCs were removed from transwell insert, treated with different stimulations as specified, and then analysed by flow cytometry. **(A)** Flow cytometry plot depicts surface VZV-gE:gI expression of MAIT cells following transwell co-incubation. **(B)** Flow cytometry plots depict surface CD69 expression of untreated MAIT cells co-incubated with either mock or VZV infected cells with a transwell. **(C)** Graph shows frequency of CD69 expression of mock (blue) and VZV (magenta) co-incubated MAIT cells to treatments as specified with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing CD69 expression in mock and VZV co-incubated MAIT cells within each treatment group was performed via Šídák's multiple comparisons test (n = 4). **(D,E and F)** Human PBMCs were incubated for one day with supernatants derived from mock or VZV co-culture with PBMCs. PBMCs were then treated with different stimulations as specified, and analysed by flow cytometry. **(D)** Flow cytometry plot depicts surface VZV-gE:gI expression of MAIT cells incubated with VZV-PBMC derived supernatant. **(E)** Flow cytometry plots depict surface CD69 expression of untreated MAIT cells incubated with either mock-PBMC or VZV-PBMC co-culture derived supernatant. **(F)** Graph shows frequency of CD69 expression of mock (green) and VZV (purple) supernatant incubated MAIT cells to treatments as specified with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing CD69 expression of mock or VZV supernatant incubated MAIT cells within each treatment group was performed via Šídák's multiple comparisons test (n = 6). *$p<0.05$, **$p<0.01$.

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cell line) were loaded with partially fixed *E. coli* for two hours, with the addition of either MR1 blocking antibody or respective isotype control antibody in the final hour of loading to control for TCR driven activation of MAIT cells. Mock or VZV inoculated PBMCs were then co-cultured with loaded THP-1 cells for 6 hours. Compared to both untreated and MR1 blocking condition, *E. coli* treated mock MAIT cells demonstrated a 51-fold increase in CD107a expression (Fig 5A and 5B). Contrastingly, VZV infected MAIT cells maintained consistent expression of CD107a across all treatment conditions, with no observed upregulation with *E. coli* treatment (Fig 5B). Again, *E. coli* treatment induced the greatest expression of Granzyme B in mock MAIT cells (19.6-fold increase compared to untreated), whilst this upregulation was completely absent in VZV infected MAIT cells (Fig 5C). Without stimulation, mock MAIT cells expressed perforin (mean 22.1%, untreated condition), which increased in both the MR1 blocking condition (mean 29.9%) and *E. coli* treatment (mean 44.5%) (Fig 5D). Conversely, VZV infected cells also expressed perforin in unstimulated cells (mean 19.6%, untreated

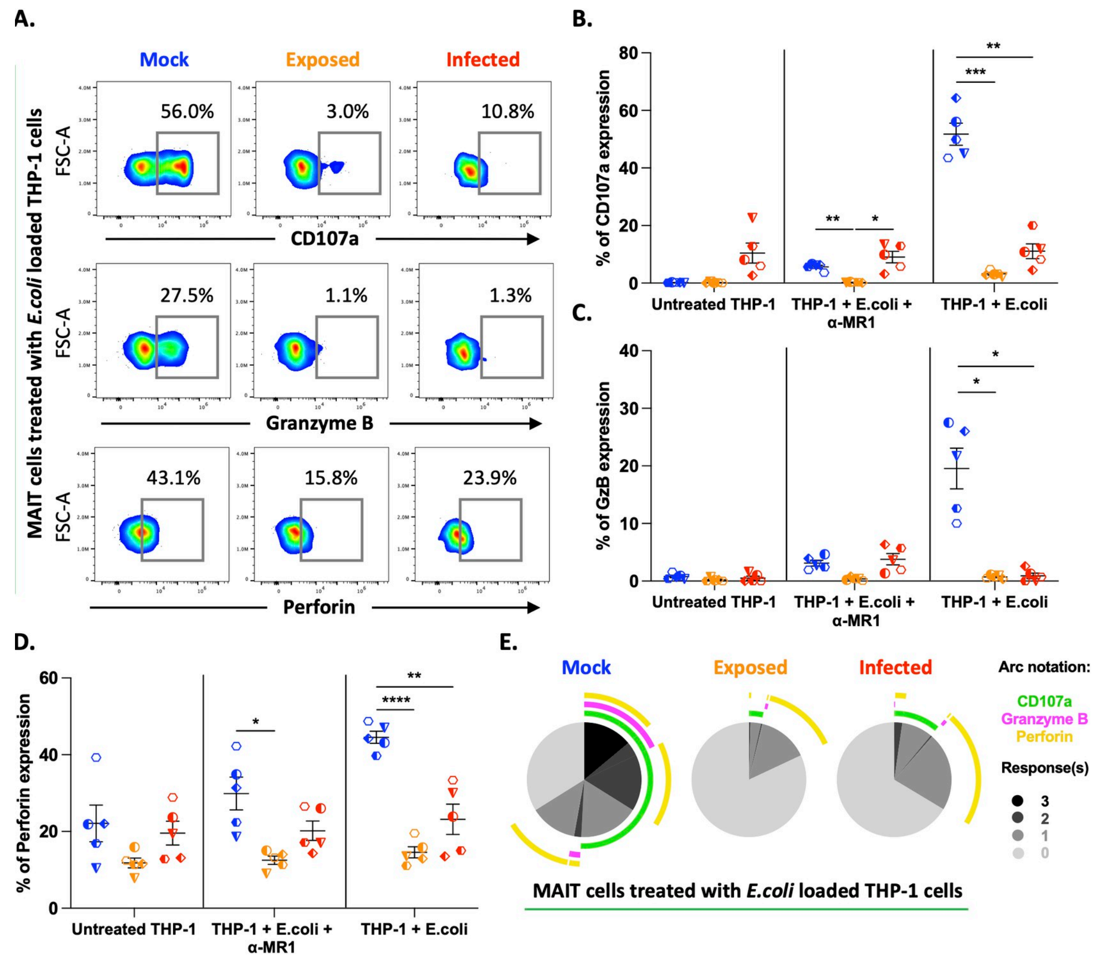

Fig 5. VZV impairs MAIT cell cytolytic potential towards bacterially stimulated target cells. (A,B,C,D and E) Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for one day. PBMCs were removed from co-culture, before treating with different stimulations as specified, and then analysed by flow cytometry. **(A)** Flow cytometry plots depict surface expression of CD107a and intracellular expression of granzyme B and perforin by mock, exposed and infected MAIT cells in response to treatment with *E. coli* loaded THP-1 cells. **(B,C and D)** Graphs show frequency of CD107a **(B)**, Granzyme B **(C)** and Perforin **(D)** expression of mock (blue), exposed (orange) and infected (red) MAIT cells to treatments as specified with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing CD107a **(B)**, Granzyme B **(C)** and Perforin **(D)** expression between mock, exposed and infected MAIT cells within each treatment group was performed via Two-Way repeated measures ANOVA (n = 6). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **(E)** SPICE pie charts show the proportion of responses by mock, exposed and infected MAIT cells to *E. coli* loaded THP-1 cells based on the combinations of CD107a, Granzyme B, and perforin expression. Pie slices indicate the number of responses (0–3) (key, bottom right). Arcs depict the markers detected for each response (key, top right). SPICE data represents the mean of five donors.

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condition) however, this did not increase with *E. coli* treatment (mean 23.6%) or MR1 blockade (mean 20.2%) (Fig 5D). VZV exposed MAIT cells exhibited the greatest inhibition of CD107a and Granzyme B expression in all treatment conditions (Fig 5B and 5C). Interestingly, VZV exposed MAIT cells expressed lower perforin at when unstimulated (mean 11.8%, untreated condition), and demonstrated a failure to upregulate across stimulation conditions (Fig 5D). Strikingly, SPICE analysis revealed that perforin expression in *E. coli* treated, VZV exposed and infected MAIT cells did not correspond to CD107a expression, therefore suggesting a lack of degranulation and release of perforin (Fig 5E). Overall, we observed defective cytolytic potential of VZV exposed and infected MAIT cells to intact bacterial presence.

Discussion

This study identifies a multifaceted immune evasion strategy utilised by VZV to restrict MAIT cell responses to TCR dependent and TCR-independent stimuli. Specifically, VZV was found to compromise activation, cytokine expression and cytolytic capacity of human MAIT cells.

Successful infection of the host involves effectively managing several complex trans-kingdom interactions between the pathogen, host and resident microbiome. Varicella manifests as cutaneous vesicular lesions, which disrupt the normal skin architecture at these sites within the host. Importantly, this potentially permits translocation of MAIT cell-activating resident riboflavin-synthesising microbes. Indeed, the most common complication from severe VZV infection is secondary bacterial infection from species such as *Staphylococcus aureus* (*S.aureus*) [13,43]. Within this co-localised microenvironment of active riboflavin biosynthesis and viral replication, MAIT cells can rapidly produce pro-inflammatory cytokines, including IFN-γ and TNF, which poses a potential threat to VZV pathogenesis. This is exemplified by several reports that demonstrate that IFN-γ and TNF mediated control of VZV replication and spread [44]. Indeed, recent reports also describe increased reactivation of VZV in patients undergoing anti-TNF treatment [45]. Remarkably, we found that both VZV exposed and infected populations targetted several effector arms of MAIT cells such as pro-inflammatory cytokine expression and cytolytic potential in response to both TCR dependent and cytokine dependent stimulation. Therefore, revealing a profound ability of VZV to suppress MAIT cell polyfunctional responses towards several distinct modalities of stimulation.

Furthermore, we demonstrated this impairment of MAIT cell responses to not only the purified MAIT TCR ligand, but also in response to intact riboflavin- synthesising bacteria. Given our previous report characterising VZV modulation of MR1 antigen presentation [37], it is possible that the restriction of TCR driven MAIT cell functionality could be indirectly attributed to an impaired ability of surrounding APCs in the PBMC-inoculum co-culture system to present 5-OP-RU. However, the functional inhibition of MAIT cells in response to exogenously added THP-1 cells pre-loaded with *E.coli* strongly suggests VZV dysregulation of TCR driven MAIT cell response to be a direct consequence of exposure and infection.

It remains to be shown whether the flow cytometry based assessment of functional cytolytic markers within this study translates to distinct functional outcomes such as impaired direct lysis of bacterially challenged APCs by exposed and infected MAIT cells. It is important to note however that without extensive antigen priming, prolonged expansion and substantial prior activation, *ex-vivo* MAIT cells are poorly cytolytic and display limited target cell killing [23,46–51]. Therefore, the extensive demands required for cytolytically arming and licensing MAIT cell killing may prove challenging due to the timescale of the infection model utilised within this current study. Thus, further studies are required to functionally illuminate the potential impairment of MAIT cell killing capacity presented within this study.

Previous landmark studies have demonstrated redirection of phosphorylation cascades in key TCR signalling proteins by VZV [8]. In particular, VZV infected tonsillar T cells exhibit increased Zap70 phosphorylation [8]; which is a critical signalling event for T cell activation following TCR engagement [52]. Interestingly, increased Zap70 phosphorylation by VZV did not result in typical downstream phosphorylation of proteins leading to cytokine production such ERK1/2, but instead non-classical phosphorylation cascades associated with cell proliferation pathways [8]. Given the conservation of TCR signalling cascade across T cells, it is likely VZV employs a similar restructuring of MAIT TCR signalling to prevent a functional response. Additionally, the closely related virus: herpes simplex virus type 1 (HSV-1) encodes protein kinase Us3 which partially inhibits TCR signalling through disrupting activation of key pathway protein LAT [53]. However, the VZV encoded homolog ORF66 is critical for T

lymphotropism [7]. Therefore, investigating the impact of ORF66 on MAIT TCR signalling presents several complexities.

Whilst no reports to date have identified a viral encoded MAIT TCR ligand, MAIT cells play a role in several viral infections by responding to cytokine production from infected host cells [34–36]. Similarly, we observed increased MAIT cell activation in response to incubation with VZV-PBMC supernatant, therefore strongly suggesting an underlying impact of VZV to also target cytokine driven activation of MAIT cells. We found that VZV infected MAIT cells remained unresponsive to IL-12/IL-18 stimulation with abrogated activation, cytokine and granzyme B production. It will be important to investigate whether VZV directly targets the expression of IL-12 and IL-18 receptor expression on MAIT cells to mediate this impairment of cytokine driven response. Previous work has demonstrated that VZV prevents the nuclear translocation of nuclear factor κB (NF-κB) in immature dendritic cells [54]. Given that both cytokine and TCR mediated stimulation of T cells converges through the NF-κB pathway [55], it is possible that VZV disruption of NF-κB translocation in MAIT cells may explain the global restriction of MAIT cell functional response towards TCR, cytokine and combination stimulation.

It is likely that a predetermined pool of VZV susceptible MAIT cells exists given that VZV antigen negative cells did not become VZV antigen positive 24 and 48 hours after sorting. This correlates with our previous finding that VZV infection of MAIT cells does not significantly increase from 24-72hpi [38]. Both findings support the notion that a predominant majority of MAIT cells do not support productive infection by VZV. This pattern of infection susceptibility has also previously been observed in studies on VZV permissiveness of NK cells and T cells [8,9]. Strikingly, despite the inability to successfully infect the majority of MAIT cells in our co-culture system, we found that VZV exposed MAIT cells consistently exhibited the greatest impairment of activation, transcription factor and functional marker expression in response to TCR dependent and TCR independent forms of stimulation. Lack of productive infection however does not preclude the possibility of direct viral contact and entry of VZV into MAIT cells. Previous studies have shown that contact between VZV inoculum and plasmacytoid dendritic cells (pDCs) impaired their ability to express IFN-α [6], whilst inhibition of NK functionality by VZV is also contact dependent [10]. These findings are congruent with HSV-1 dysregulation of TCR signalling in both immortalised T cells and invariant natural killer T (iNKT) cells, which was revealed to be dependent on viral entry not infection [56,57]. Future studies utilising anti-viral drugs such as acyclovir [58], which permit viral entry but restrict *de novo* viral gene expression, will shed light on whether suppression of MAIT cell response in VZV exposed cells is mediated by viral entry.

There are now several reports that characterise decreased functional capacities of MAIT cells as an outcome of various infections. Increased conjugated bilirubin levels released from liver damage during chronic HBV infection drives exhaustion and impairs TCR driven MAIT cell activation and proliferation [59]. Altered MAIT cell phenotypes observed during other liver damaging disease such as HCV infection [60] suggests that increases in conjugated bilirubin levels may drive this MAIT cell disruption as seen in conventional $CD4^+$ T cells [61]. Studies have now also reported a persistent decline and exhaustion of MAIT cells during HIV infection [62,63]. HIV infection drives sustained IFN-α expression consequently inducing a counter-balancing IL-10 driven immune-suppressive response by monocytes [64]. Critically, this increased IL-10 production impairs TCR driven CD107a and granzyme expression by MAIT cells in response to *E. coli* stimulation [64]. Furthermore, a recent study demonstrated activation induced pyroptosis of MAIT cells in response to HIV virions [63], which may potentially explain the consistent observation of declining circulating MAIT cells in HIV patients.

Overall, there is a growing appreciation that MAIT cells play an important role in the control of bacterial and viral infections. Our study characterises a direct immunoevasive strategy employed by a pathogen to counteract MAIT cell responses. Specifically, we demonstrate that exposure to, and infection by, VZV causes a profound suppression of MAIT cell activation and functional response, both to TCR-dependent and -independent forms of stimulation. Future mechanistic insights into the restriction of MAIT cell responses by VZV could potentially be harnessed for bio-active therapeutic alternatives in treating chronic inflammatory settings such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel diseases where MAIT cells play an immunopathological role [65]. The conserved nature of MAIT cell activation and downstream functional response drives a selective evolutionary pressure for a diverse range of human pathogens. Therefore, it is likely that the restriction of MAIT cell response is not a strategy unique to VZV, but one that is potentially also employed by other pathogens to varying degrees. Thus, our findings predicate an exploration into how pathogens can directly control and compromise MAIT cell mediated host responses.

Materials and methods

Ethics statement

All blood work was performed in the accordance with The University of Sydney Human Research Ethics Committee approval. All blood donations were obtained under agreement with the Australian Red Cross Lifeblood service and all donors provided written informed consent.

Human blood

Healthy peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare). All buffy coats were de-identified, so any comparative data on sex and age is not available. For long term storage, PBMCs were preserved with liquid nitrogen in freezing media (90% Fetal Calf serum (FCS)) (Sigma-Aldrich) with 10% DMSO.

Cell lines

ARPE-19 epithelial cells (ATCC) were maintained in complete DMEM (Lonza) supplemented with 10% FCS and 1% Penicillin Streptomycin (P/S) (ThermoFisher). THP-1 (human monocyte cell line, ATCC) were maintained in complete RPMI 1640 (ThermoFisher Scientific), supplemented with 10% FCS and 1% P/S. All cell lines were cultured at 37˚C, 5% CO_2.

Short term culture of PBMCs and isolation of MAIT cells

PBMCs were thawed and washed in FCS supplemented RPMI before being maintained RPMI supplemented with 10% human serum (Sigma-Aldrich) and 1% P/S. MAIT cells were identified through fluorescent co-staining of CD3 and MR1-Tetramer positive and FACS isolated from whole PBMCs. MAIT cells were sorted to a >95% purity using BD FACSMelody (BD Biosciences) and then maintained in RPMI supplemented with 10% human serum (H/S) and 1% P/S. Where specified, VZV gE:gI antigen negative MAIT cells were also isolated to a >90% purity using BD FACSMelody (BD Biosciences) and then maintained in RPMI supplemented with 10% human serum (H/S) and 1% P/S for 24–48 hours.

VZV inoculation of PBMCs

PMBCs were either mock or VZV inoculated through co-culture with either uninfected (mock inoculum) or VZV infected ARPE-19 cells (VZV inoculum), respectively. VZV inoculum was

trypsinised after demonstrating >75% cytopathic effect and added to PBMCs at a ratio of 1:5 (ARPE-19:PBMC). Infections were performed in 12 well plates, with $2x10^6$ PBMCs in 2ml of complete RPMI medium per well. Following the addition of either mock or VZV inoculum to PBMCs, cells were spinoculated for 15 minutes at 150 x *g*, 37˚C. Plates were then incubated at 37˚C, 5% CO_2 for 24 hours.

For transwell experiments, mock and VZV inoculum was seeded into the bottom chamber of a 6 well plate and separated from the top chamber containing PBMCs by a 0.4μM pore polycarbonate transwell membrane (Corning). Plates were then incubated at 37˚C, 5% CO_2 for 24 hours.

Treatment of PBMCs with mock and viral co-culture supernatants

Following 24 hours of mock and VZV inoculation of PBMCs, supernatant was collected, spun at 460 x *g* to remove cells, and then frozen at -80˚C. When required, supernatant was thawed, and diluted 1:1 with fresh complete RPMI (+10% H/S and 1% P/S) before addition to PBMCs for 24 hours.

Preparation of bacterial stimulation

Escherichia coli (E. coli) DH5α was grown overnight in Luria-Bertani (LB) broth, washed with PBS and then partially fixed in 1% paraformaldehyde (PFA) for 3 minutes with vortexing in first 60 seconds and last 30 seconds of the fixation. After extensive washing, *E. coli* was added to THP-1 cells at 30 bacteria per cell (BpC) for whole PBMC stimulation assays.

5-OP-RU

The MAIT cell antigen 5-OP-RU was synthesised as a solution in DMSO, and its concentration quantified by NMR and MS spectra, as previously described [66]. While it is chemically stable in DMSO, and when bound to MR1 in solution, care should be taken to restrict exposure times to water during handling and dilution since 5-OP-RU rapidly transforms in aqueous media ($t_{1/2}$ 1h, 37˚C) to much less active lumazines [66]. Thus, PBMCs were treated with 5-OP-RU immediately after defrosting.

In vitro MAIT cell stimulations

24 hours post-mock or -VZV inoculation, PBMCs were removed from the inoculum monolayer by gently washing off with PBS and seeded in U-bottom 96 well plates at a concentration of $1x10^6$ PBMCs/200 ml. The following treatment condition concentrations and timepoints were chosen in line with previous literature [22]. For MAIT TCR specific triggering, PBMCs were treated with 5-OP-RU (10 nM) for 6 hours. For cytokine dependent stimulation, PBMCs were treated with 50 ng/mL of IL-12 (Miltenyi) and 50 ng/mL of IL-18 (R&D Systems) for 24 h. In the combination stimulation condition, PBMCs were treated with 5-OP-RU (10 nM) and IL-12+IL-18 (each at 50 ng/mL) for 24 h. PBMCs were treated with DMSO in the untreated condition for 24 h. For stimulation with whole bacteria, THP-1 cells were pulsed with partially fixed *E.coli* (30 BpC) for 2 h, with the addition of either MR1 blocking antibody (5 μg) or respective isotype control (5 μg) in the final hour of pulsing. Loaded THP-1 cells were then added to PBMCs at a ratio of 1:5 (THP-1:PBMC). For assessment of cytokine content, Brefeldin A (BFA) (Biolegend) was added at 5 μg/mL for the final 4 h of the stimulation. For observation of degranulation, BFA (5 μg/mL) and Monensin (5 μg/mL) (Biolegend) was added together for the final 4 h of stimulation.

Flow cytometry

Cells were viability stained with Live/Dead Aqua (Invitrogen), and then stained on ice for 45 minutes for the following markers: CD3—BUV395 (clone UCHT-1, BD Biosciences), CD69—PerCP Cy5.5 (clone FN50, Biolegend), PD-1—PE/Dazzle (clone EH12.2H7, Biolegend), CD161- PE/Dazzle 594 (clone HP-3G10, Biolegend), Vα 7.2—PE/Cy7 (clone 3C10, Biolegend), VZV gE:gI—Dy488 (clone SG1-1, Meridian Life Sciences), MR1-Tetramer—PE (kindly provided by A/Prof. Alexandra Corbett, University of Melbourne). Cells were then fixed in 4.2% Cytofix/Cytoperm (BD Biosciences) at room temperature for 30 minutes before staining with intracellular markers: granzyme B—PE/Cy7 (clone QA16A02), IFN-γ - BV421 (clone B27), TNF—BV785 (clone Mab11), perforin—APC/Cy7 (clone DG9); all Biolegend, Caspase-3—PE (CC3) (clone C92-605, BD Biosciences). For assessment of transcription factors, cells were permeabilized with Foxp3/transcription buffer set (ThermoFisher Scientific) and then stained for: T-bet—BV421 (clone 4B10, Biolegend) and RORγt–APC (clone AFKJS-9, ThermoFisher Scientific). For degranulation staining, fluorescently conjugated anti-CD107a-APC (clone H4A3, Biolegend) or respective isotype control (Biolegend) was added to culture for the duration of stimulation. All samples were acquired on the Cytek Aurora 5 laser cytometer (Cytek), and then analysed using FlowJo v10 (TreeStar). Following exclusion of dead cells (as per viability staining) and morphological gating of lymphocytes (as per characteristic forward and side scatter), MAIT cells were identified as CD3 and MR1-Tetramer positive (Fig 1A).

Quantification and statistical analysis

All graphs and statistical analyses were performed using GraphPad Prism V10.02. Statistical significance of marker expression between mock, exposed and infected MAIT cells was assessed using two-way repeated-measures ANOVA or paired two tailed *t* tests.
Mean ± standard error of mean (SEM) is shown throughout.

Supporting information

S1 Fig. MAIT cell frequency and rate of infection across treatment conditions. Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for one day. PBMCs were removed from co-culture, before treating with different stimulations as specified, and then analysed by flow cytometry. **(A)** Graph shows frequency of MAIT cells of live T cells after mock (blue) and VZV (red) inoculation, with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing MAIT cell frequency between mock and VZV inoculation was performed via paired *t* test (n = 19). **(B)** Graph shows frequency of gE:gI expression by MAIT cells across treatment conditions as specified, with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing gE:gI expression by MAIT cells between treatment conditions was performed via repeated measures one-way ANOVA (n = 10). *$p<0.05$, **$p<0.01$, ***$p<0.001$.
(TIF)

S2 Fig. VZV exposed or infected MAIT cells do not exhibit significantly greater levels of apoptosis. Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for one day. PBMCs were removed from co-culture, washed and then viability stained with Live/Dead dye. Following Live/Dead staining, PBMCs were stained for surface markers and then permeabilised with 4.2% Cytofix/Cytoperm (BD Biosciences) at room temperature for 30 minutes. Following permeabilization, PBMCs were intracellular stained for cleaved Caspase-3 (CC-3) at room temperature for 1 hour. Intracellular CC-3

expression was assessed by flow cytometry. **(A)** Flow cytometry plots depicts co-staining of Live/Dead dye with intracellular expression of CC-3 in mock, exposed and infected MAIT cells. **(B)** Graph shows frequency of CC-3 expression of mock (blue), exposed (orange) and infected (red) MAIT cells, with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing CC-3 expression between mock, exposed and infected MAIT cells was performed via Two-Way repeated measures ANOVA (n = 5). $^{*}p<0.05$, $^{**}p<0.01$.
(TIF)

S3 Fig. Most gE:gI negative MAIT cells remain gE:gI negative. Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for one day. PBMCs were removed from co-culture, FACS sorted for gE:gI negative MAIT cells and then cultured for 24 and 48 hours. **(A)** Representative flow cytometry plots depict the gating strategy to sort VZV exposed MAIT cells in PBMCs as $CD3^{+}$/ MR1-Tetramer^{+}/ gE:gI^{-}. (B) Graph shows frequency of gE:gI expression by gE:gI negative isolated MAIT cells 24 and 48 hours post sorting. Symbols representing individual donors and mean and SEM indicated by the bars (n = 3).
(TIF)

S4 Fig. VZV impairs MAIT cell response across several infectious dose ratios. Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells at varying inoculum: PBMC ratios as specified for 24 hours. PBMCs were removed from co-culture, before treating with different stimulations as specified, and then analysed by flow cytometry. **(A)** Graph shows frequency of gE:gI expression by MAIT cells across various inoculum: PBMC ratios for different treatment conditions as specified, with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing gE:gI expression by MAIT cells between different inoculum: PBMC ratios within each treatment condition was was performed via repeated measures one-way ANOVA (n = 3). $^{*}p<0.05$, $^{**}p<0.01$. **(B)** Flow cytometry plots depict co-expression of surface CD69 and intracellular IFN-γ for mock, exposed and infected MAIT cells in response to 5-OP-RU + IL-12/IL-18 treatment at the 1:20 inoculum: PBMC ratio. **(C)** Graphs show frequency of CD69 and IFN-γ co-expression of mock (blue), exposed (orange) and infected (red) MAIT cells to different treatment conditions for the various inoculum: PBMC ratios, with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing CD69 and IFN-γ co-expression expression of exposed and infected MAIT cells at different inoculum: PBMC ratios to mock control within each treatment group was performed via Two-Way repeated measures ANOVA (n = 3). $^{*}p<0.05$, $^{**}p<0.01$.
(TIF)

S5 Fig. VZV impairs MAIT cell polyfunctional response. Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for one day. PBMCs were removed from co-culture, before treating with different stimulations as specified, and then analysed by flow cytometry. **(A)** Flow cytometry plots depict intracellular co-expression of IFN-γ and TNF of mock, exposed and infected MAIT cells in response to 5-OP-RU and 5-OP-RU + IL-12/IL-18. **(B)** Graph show frequency of IFN-γ and TNF co-expression of mock (blue), exposed (orange) and infected (red) MAIT cells to treatments as specified with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing IFN-γ and TNF co-expression between mock, exposed and infected MAIT cells within each treatment group was performed via Two-Way repeated measures ANOVA (n = 7). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. **(C)** SPICE pie charts show the proportion of

responses by mock, exposed and infected MAIT cells to 5-OP-RU stimulation, based on the combinations of granzyme B, IFN-γ and TNF expression. Pie slices indicate the number of responses (0–3) (key, bottom right). Arcs depict the markers detected for each response (key, top right). SPICE data represents the mean of seven donors.
(TIF)

S1 Data. Minimal data set.
(XLSX)

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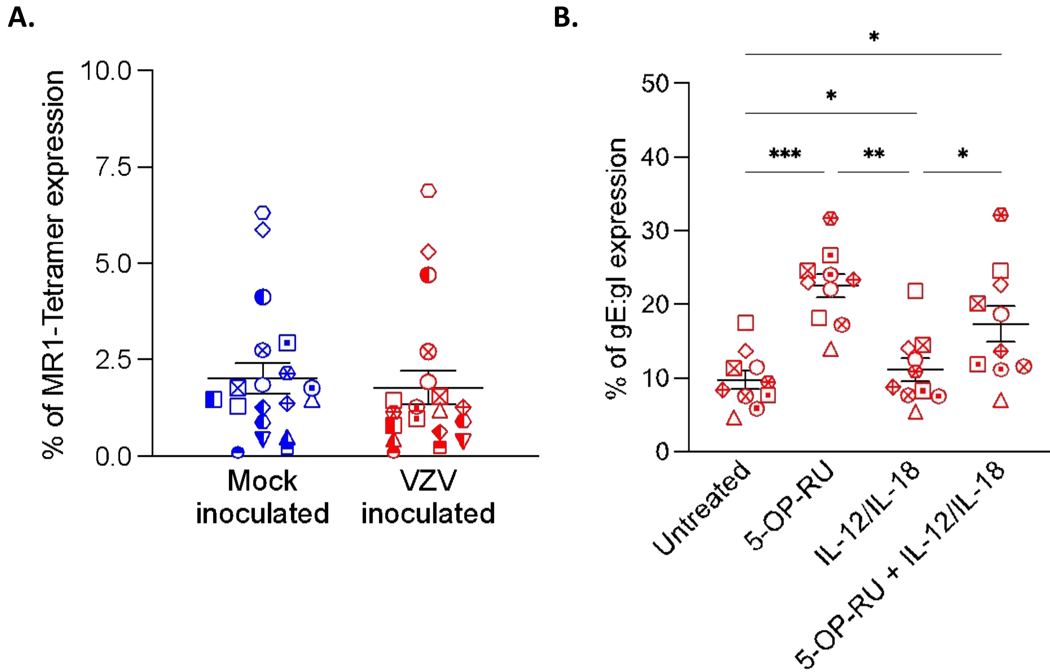
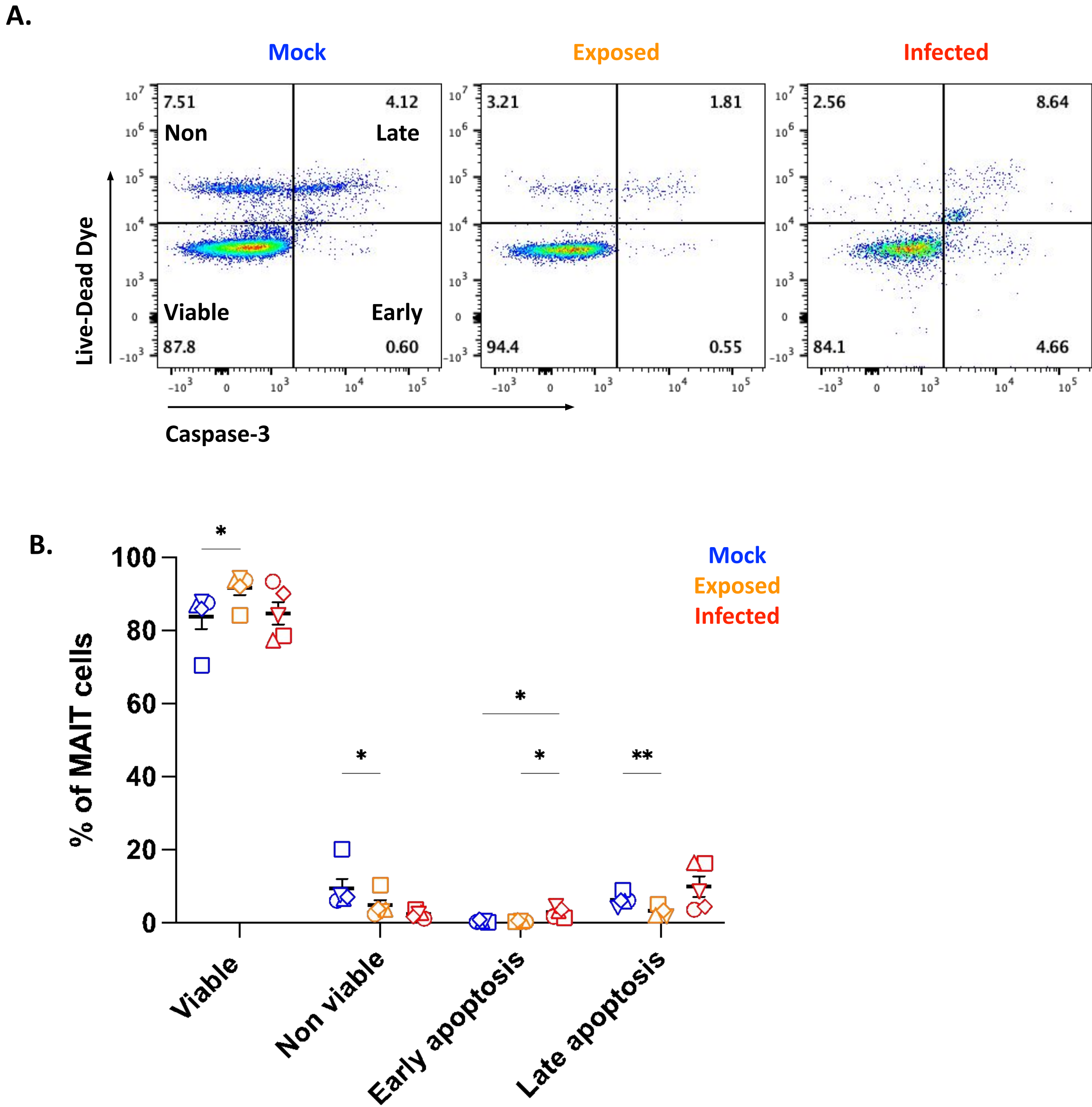


S1 Fig. MAIT cell frequency and rate of infection across treatment conditions.

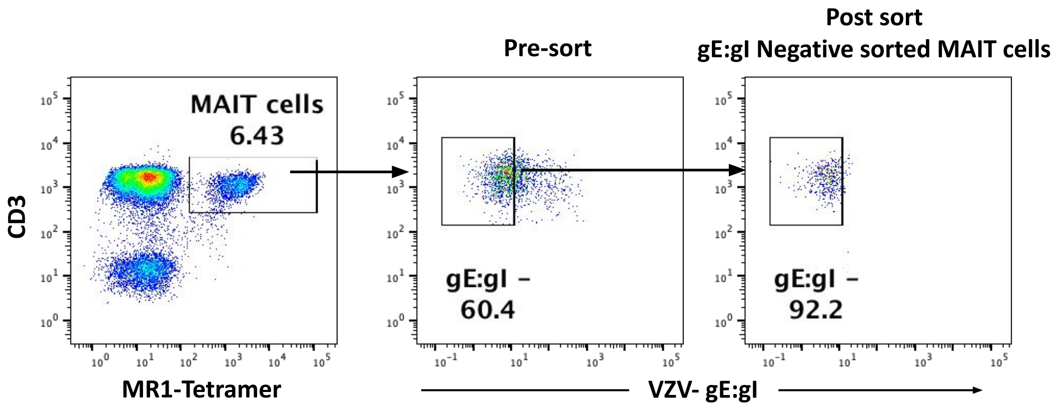
Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for one day. PBMCs were removed from co-culture, before treating with different stimulations as specified, and then analysed by flow cytometry. **(A)** Graph shows frequency of MAIT cells of live T cells after mock (blue) and VZV (red) inoculation, with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing MAIT cell frequency between mock and VZV inoculation was performed via paired *t* test (n = 19). **(B)** Graph shows frequency of gE:gI expression by MAIT cells across treatment conditions as specified, with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing gE:gI expression by MAIT cells between treatment conditions was performed via repeated measures one-way ANOVA (n = 10). *p<0.05, **p<0.01, ***p<0.001.



[S2 Fig.](#) **VZV exposed or infected MAIT cells do not exhibit significantly greater levels of apoptosis.**

Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for one day. PBMCs were removed from co-culture, washed and then viability stained with Live/Dead dye. Following Live/Dead staining, PBMCs were stained for surface markers and then permeabilised with 4.2% Cytofix/Cytoperm (BD Biosciences) at room temperature for 30 minutes. Following permeabilization, PBMCs were intracellular stained for cleaved Caspase-3 (CC-3) at room temperature for 1 hour. Intracellular CC-3 expression was assessed by flow cytometry. **(A)** Flow cytometry plots depicts co-staining of Live/Dead dye with intracellular expression of CC-3 in mock, exposed and infected MAIT cells. **(B)** Graph shows frequency of CC-3 expression of mock (blue), exposed (orange) and infected (red) MAIT cells, with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing CC-3 expression between mock, exposed and infected MAIT cells was performed via Two-Way repeated measures ANOVA (n = 5). *p<0.05, **p<0.01.

A.



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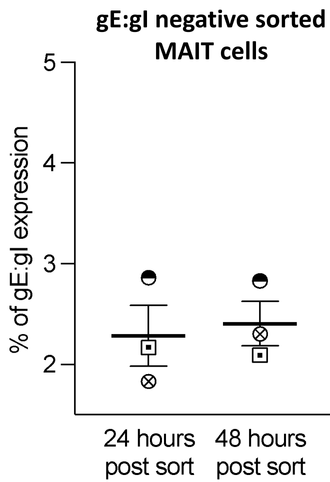
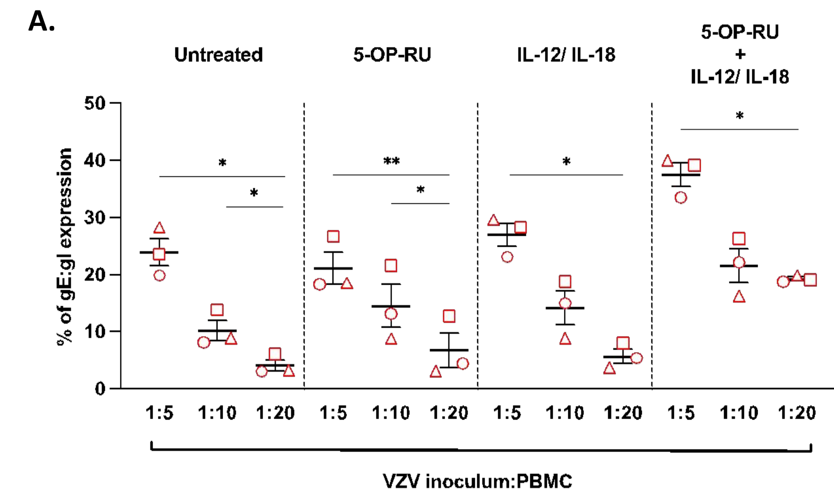
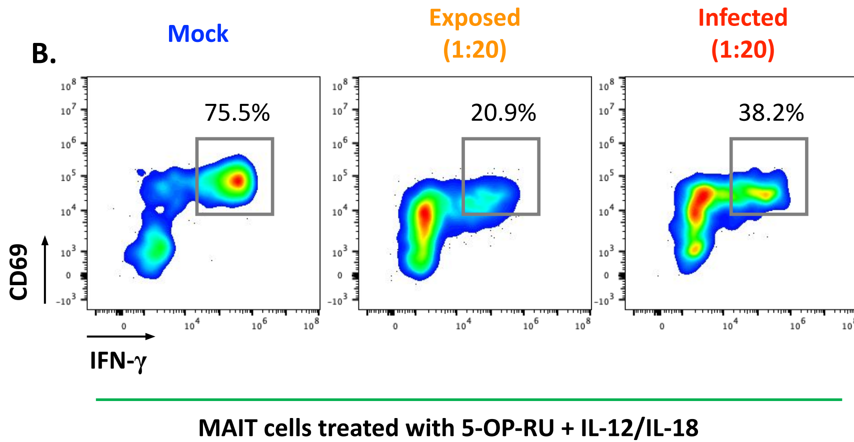
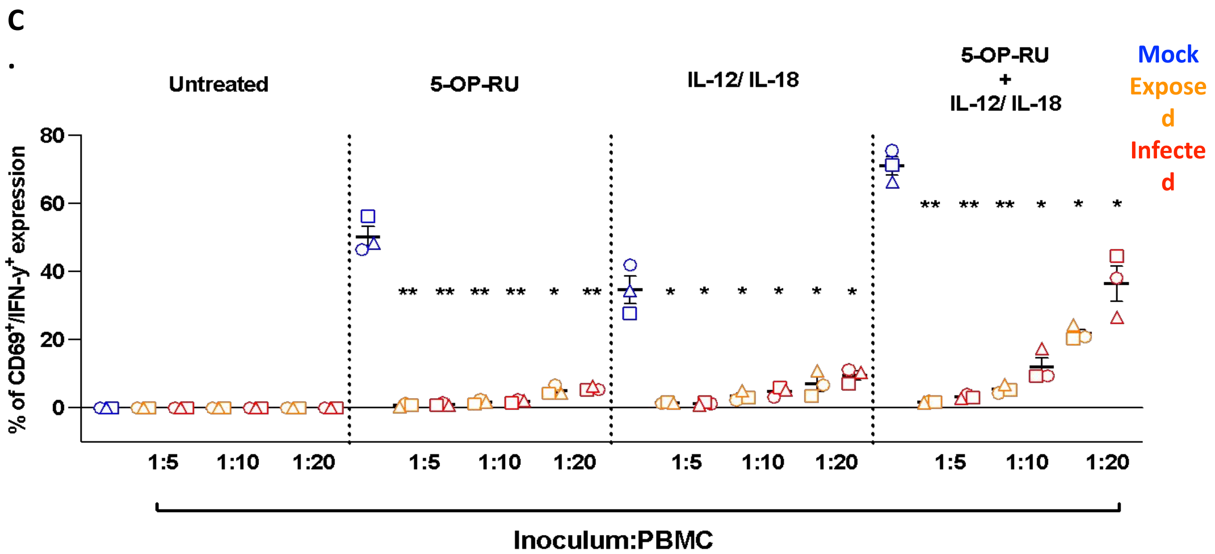


S3 Fig. Most gE:gI negative MAIT cells remain gE:gI negative.

Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for one day. PBMCs were removed from co-culture, FACS sorted for gE:gI negative MAIT cells and then cultured for 24 and 48 hours. **(A)** Representative flow cytometry plots depict the gating strategy to sort VZV exposed MAIT cells in PBMCs as CD3+/ MR1-Tetramer+/ gE:gI-. (B) Graph shows frequency of gE:gI expression by gE:gI negative isolated MAIT cells 24 and 48 hours post sorting. Symbols representing individual donors and mean and SEM indicated by the bars (n = 3).

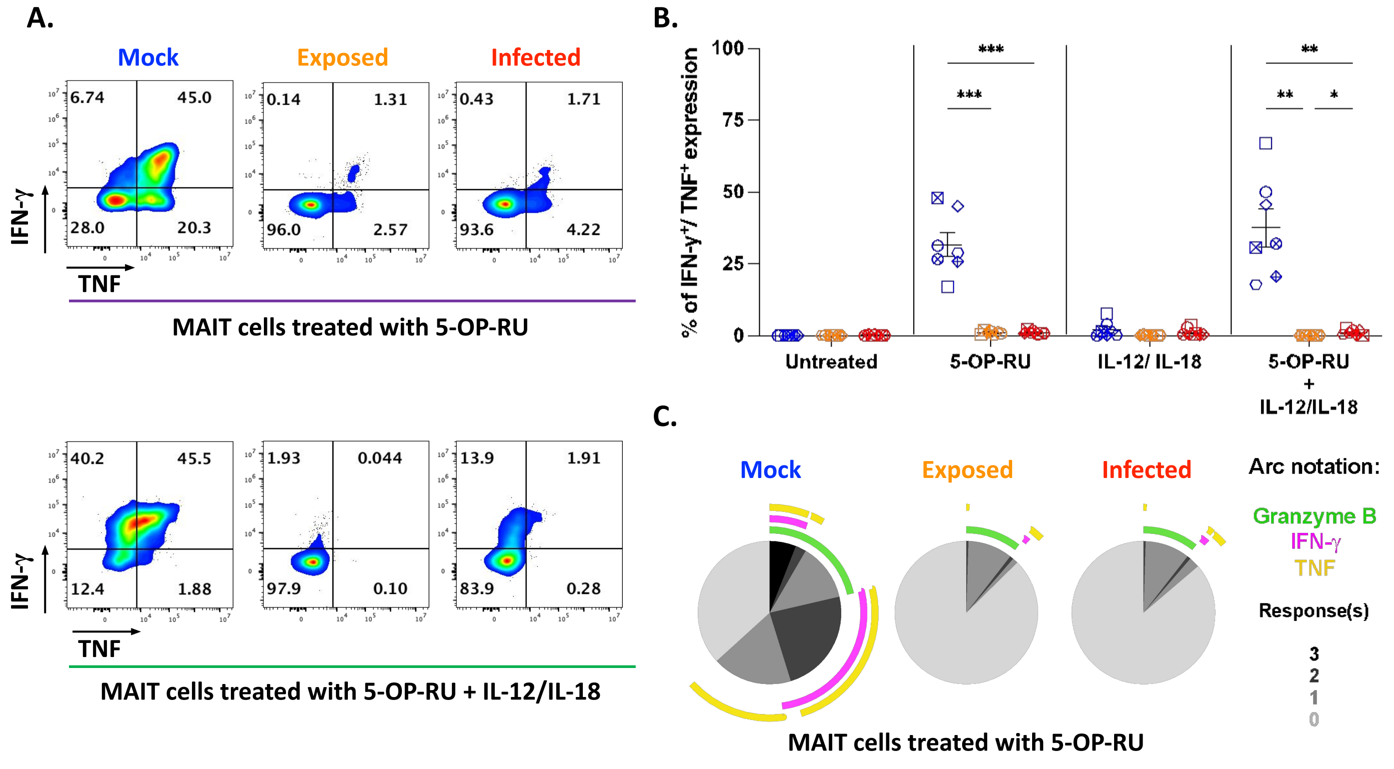






S4 Fig. VZV impairs MAIT cell response across several infectious dose ratios.

Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells at varying inoculum: PBMC ratios as specified for 24 hours. PBMCs were removed from co-culture, before treating with different stimulations as specified, and then analysed by flow cytometry. **(A)** Graph shows frequency of gE:gI expression by MAIT cells across various inoculum: PBMC ratios for different treatment conditions as specified, with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing gE:gI expression by MAIT cells between different inoculum: PBMC ratios within each treatment condition was was performed via repeated measures one-way ANOVA (n = 3). *p<0.05, **p<0.01. **(B)** Flow cytometry plots depict co-expression of surface CD69 and intracellular IFN-γ for mock, exposed and infected MAIT cells in response to 5-OP-RU + IL-12/IL-18 treatment at the 1:20 inoculum: PBMC ratio. **(C)** Graphs show frequency of CD69 and IFN-γ co-expression of mock (blue), exposed (orange) and infected (red) MAIT cells to different treatment conditions for the various inoculum: PBMC ratios, with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing CD69 and IFN-γ co-expression expression of exposed and infected MAIT cells at different inoculum: PBMC ratios to mock control within each treatment group was performed via Two-Way repeated measures ANOVA (n = 3). *p<0.05, **p<0.01.



S5 Fig. VZV impairs MAIT cell polyfunctional response.

Human PBMCs were inoculated with mock or clinical VZV isolate (VZV-S) infected ARPE-19 epithelial cells for one day. PBMCs were removed from co-culture, before treating with different stimulations as specified, and then analysed by flow cytometry. **(A)** Flow cytometry plots depict intracellular co-expression of IFN-γ and TNF of mock, exposed and infected MAIT cells in response to 5-OP-RU and 5-OP-RU + IL-12/IL-18. **(B)** Graph show frequency of IFN-γ and TNF co-expression of mock (blue), exposed (orange) and infected (red) MAIT cells to treatments as specified with symbols representing individual donors and mean and SEM indicated by the bars. Statistical analysis comparing IFN-γ and TNF co-expression between mock, exposed and infected MAIT cells within each treatment group was performed via Two-Way repeated measures ANOVA (n = 7). *p<0.05, **p<0.01, ***p<0.001. **(C)** SPICE pie charts show the proportion of responses by mock, exposed and infected MAIT cells to 5-OP-RU stimulation, based on the combinations of granzyme B, IFN-γ and TNF expression. Pie slices indicate the number of responses (0–3) (key, bottom right). Arcs depict the markers detected for each response (key, top right). SPICE data represents the mean of seven donors.

Chapter 5. Discussion

5.1 Introductory statement

The host MR1-MAIT cell axis can rapidly respond to barrier disruption by detecting riboflavin synthesis conserved across diverse bacterial and fungal microbes. Whilst viruses do not synthesise riboflavin, barrier disruption during productive varicella infection could promote translocation of riboflavin synthesising microbes and consequent MR1 dependent activation of MAIT cells (Figure 1). Therefore, resulting in a pro-inflammatory co-localised environment that is likely unfavourable for viral replication. This potential interplay therefore suggested a fascinating and previously undescribed host-pathogen interaction that warranted comprehensive investigation. Initially, we demonstrated that VZV targets the intracellular reservoir of immature MR1 which impairs surface expression of MR1 (Purohit et al., 2021). Furthermore, we found that VZV productively infects circulating MAIT cells and transmits infectious virions to epithelial cells (Purohit et al., 2023). Finally, we revealed that VZV exposed and infected MAIT cells are functionally refractory to several distinct modes of activation stimuli (Purohit et al., 2024). Altogether, the findings culminated over my doctoral dissertation strongly indicates a profound paralysis of the MR1-MAIT cell axis during VZV infection (Figure 1). This concluding discussion will contextualise the findings from this thesis into biologically relevant frameworks, as well as offer insights into new avenues of research that these novel findings promote.

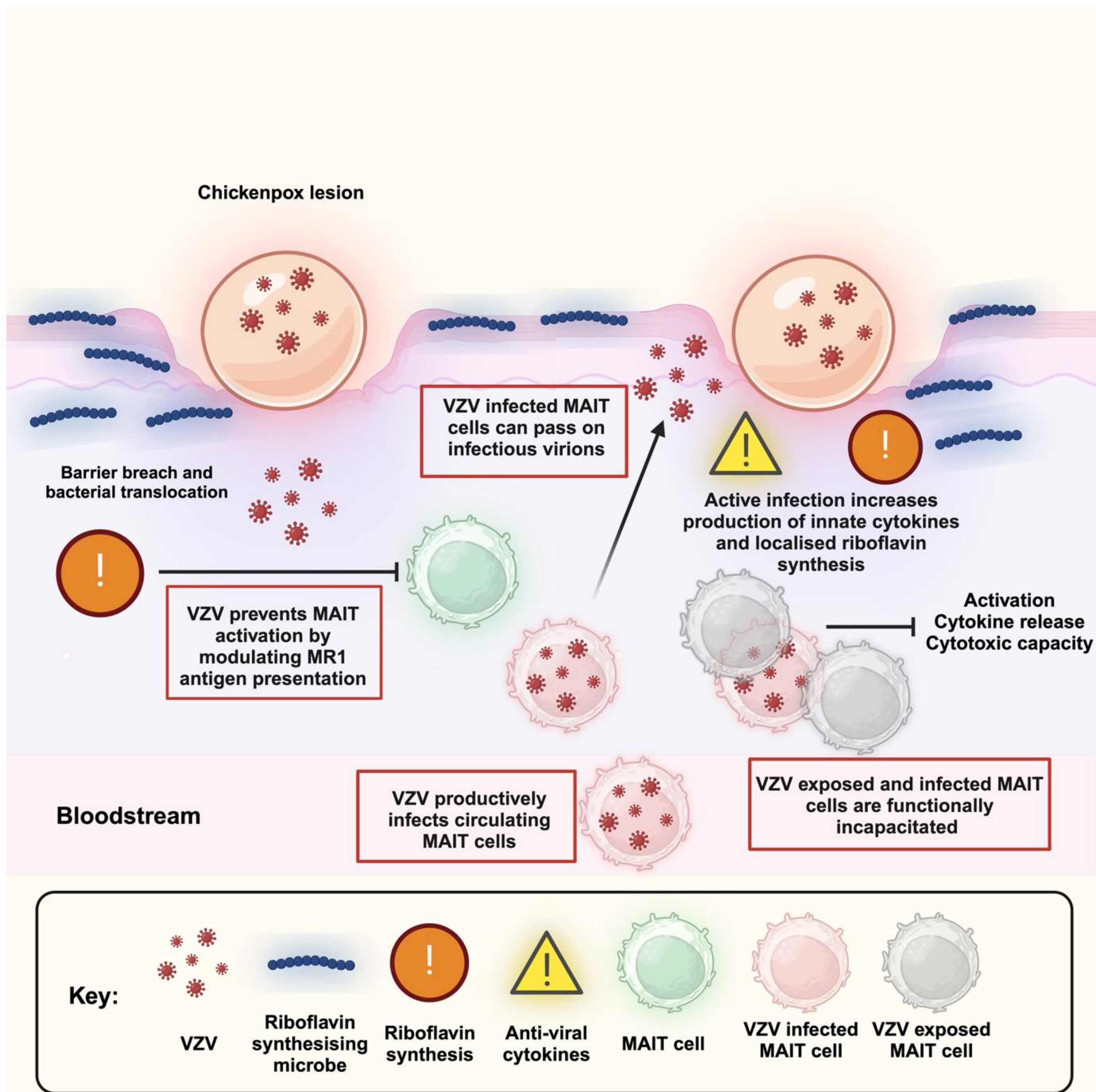


Figure 1. Proposed model of VZV suppression of the MR1-MAIT cell axis
Primary or reactivated VZV infection causes disruption of the skin architecture, which allows for translocation of riboflavin synthesising resident microbes. Increased riboflavin synthesis triggers resident MAIT cell TCR dependent response which VZV subverts by limiting MR1 expression. VZV also infects circulating MAIT cells and utilises them for transmitting infectious virions to epithelial sites. Active viral infection of epithelial sites generates production of anti-viral cytokines as well as riboflavin synthesising bacterial translocation. VZV exposed and infected MAIT cells remain functionally paralysed in response to both TCR dependent or cytokine dependent stimulation. Key findings from this thesis are indicated within the red text boxes.
Figure generated using Biorender.

5.2 VZV modulation of MR1 (Chapter 2)

Investigations carried out in the second chapter of this thesis identified a novel immunomodulatory function whereby VZV suppresses MR1 expression. We found that VZV effectively targets intracellular reservoirs of immature MR1, whilst mature ligand bound MR1 remains impervious to downregulation and retained at the cell surface throughout viral infection. This delicate balance highlighted a temporally sensitive relationship between infection timing and MR1 ligand availability. Furthermore, ectopic single gene expression of the VZV encoded serine/threonine kinase ORF66 downregulated MR1 expression. However, the downregulation of MR1 observed during infection with an ORF66 VZV mutant virus revealed that there was likely to be involvement of additional viral proteins(s) that function to suppress MR1.

Whilst we were able to identify ORF66 as partially involved in MR1 downregulation, the mechanism of action generating this suppressive phenotype remains unclear. Utilising a ORF66 kinase "dead" VZV mutant would demonstrate whether the modulation of MR1 is kinase dependent. This approach was similarly used to reveal that ORF66 downregulates MHC-I expression through both kinase dependent and independent mechanisms (Eisfeld et al., 2007a). More recently, our group also utilised kinase dead protein constructs in the context of HSV-1 encoded kinase US3 to reveal that kinase activity of US3 is only partially required for the modulation of MR1 surface expression (Samer et al., 2024a)

It is unlikely however that ORF66 modulates MR1 through a direct interaction given the lack of consensus phosphorylation sites present on MR1 which match putative ORF66 target sequences (Erazo et al., 2011). Therefore, it is more plausible that

ORF66 phosphorylates upstream cellular proteins that result in a downstream regulation of MR1 expression. This theory is not without precedence as the expression of ORF66 homolog US3 encoded by closely related alphaherpesvirus HSV-1 phosphorylates host motor protein KIF3A resulting in downstream inhibition of CD1d surface expression (Yuan et al., 2006, Rao et al., 2018, Xiong et al., 2015).

With regards to upstream modulation of MR1 expression, VZV ORF66 has been previously demonstrated to hyper-phosphorylate histone deacetylase 1 (HDAC) and HDAC 2 (Walters et al., 2009), which play a critical role in regulating chromatin accessibility (Grunstein, 1997). Therefore, it is possible that VZV may also modulate MR1 gene expression through upstream epigenetic regulation. Ultimately, a holistic phospho-proteomic study assessing and identifying all cellular ORF66 phosphorylation targets is required to comprehensively understand the mechanisms through which ORF66 regulates the MR1 antigen presentation pathway.

Furthermore, kinases ERK 1/2 were recently identified as negative regulators of MR1 gene and protein expression (Constantin et al., 2024). ERK 1/2 are kinase proteins that are part of a greater signalling cascade known as the mitogen-activated protein kinase (MAPK) pathway, which plays a central role in promoting cellular growth, differentiation and survival (Tidyman and Rauen, 2009). Whilst over-active ERK 1/2 signalling is a hallmark of tumour cells and a common cancer therapeutic target (Kohno and Pouyssegur, 2006), VZV also effectively leverages the anti-apoptotic functionality of ERK signalling to drive infection (Rahaus et al., 2006). In addition, the activation of several other anti-apoptotic cellular kinases such as Akt and P13K are a key determinant for VZV productive infection (Rahaus et al., 2007). This relationship

is particularly evidenced by patients with hyper-activated Akt syndromes who are accompanied with severe VZV infection (Cohen, 2018, Liu and Cohen, 2015). Specifically, VZV encoded ORF12 directly phosphorylates ERK 1/2 (Liu et al., 2012), Akt and P13K (Liu and Cohen, 2013) to regulate cell cycle progression and avoid apoptosis. Interestingly MAPK signalling also negatively regulates other antigen presenting pathways such as MHC-I (Stopfer et al., 2022), MHC-II (Mimura et al., 2013) and CD1d (Renukaradhya et al., 2005). Strikingly, both Vaccinia Virus and Vescicular Stromatis Virus leverage MAPK activation to inhibit CD1d expression (Renukaradhya et al., 2005). Similarly, VZV regulation of the MAPK signalling pathway may explain it's previously described ability to downregulate MHC-I (Abendroth et al., 2001a), MHC-II (Abendroth et al., 2000), and CD1d expression (Traves et al., 2023). Therefore, the downregulation of MR1 expression observed during VZV infection could be a potential side-effect derived from viral control of host cell cycle progression regulated through broadly pleiotropic kinases. Bulk transcriptomics data combined with using machine learning based pathway analysis models of ORF12 transfected cells could help untangle the intricate relationships between viral regulation of MAPK signalling and reciprocal downstream impacts on antigen presentation pathways such as MR1.

Given that viruses do not synthesise riboflavin, viral infections are not believed to generate MR1 ligands. Therefore, the driving hypothesis behind this study was that VZV targets MR1 presentation of riboflavin ligands derived from co-localised microbes. This theory holds physiological relevance given that the most common complication arising from varicella infection is secondary bacterial infection (Diniz et al., 2018, Gershon and Gershon, 2013, Welgama et al., 2011). Whilst our investigations

revealed the ability of VZV to effectively target immature MR1 in epithelial and MR1 overexpressing cell lines, these findings should be extended to primary cell lines with heightened endogenous antigen presentation capacity such as B cells, DCs and bronchial epithelial cells (McWilliam et al., 2016a, Lamichhane and Ussher, 2017, Harriff et al., 2014, Gozalbo López et al., 2009). Furthermore, through utilising established *in vitro* models of VZV latency in neuronal cells (Markus et al., 2015, Sadaoka et al., 2016, Niemeyer et al., 2024a), it can be investigated whether VZV maintains suppression of MR1 expression during latency. This is of particular interest given recent reports which describe VZV modulation of several immune pathways in 3D neurospheroid models such as MHC-II expression (Govaerts et al., 2024).

Previously, our lab has demonstrated that modulation of MR1 expression during HSV-1 and HCMV infection results in the inhibition of MAIT TCR dependent activation (McSharry et al., 2020, Ashley et al., 2023). It would be also important to investigate whether VZV mediated regulation of MR1 expression extends to inhibition of MAIT cell recognition and functionality.

In addition to microbial derived ligands, recent work by Vacchini et al, demonstrated that MR1 binds to and presents carbonyl adducts of nucleobases generated by mitochondrial stress in the context of cancer cells (Vacchini et al., 2024). The ability of MR1 to present endogenous tumour associated ligands resulted in activation and anti-tumour functional responses of MR1 restricted T (MR1T) cells (Vacchini et al., 2024); therefore correlating with earlier studies that have also reported MR1 dependent tumour recognition and killing (Crowther et al., 2020, Lepore et al., 2017, Chancellor et al., 2023). It is important to note that viruses exploit the host cell mitochondria to

control cellular respiratory output and avoid apoptotic functions. Whilst this drives viral replication, it also introduces substantial mitochondrial distress (Foo et al., 2022). In particular, changes to mitochondrial structures and progressive mitochondrial dysfunction are tightly linked to the progression of the HSV-1 replication cycle (Leclerc et al., 2024). Therefore, it is likely that VZV infection may also drive the production of mitochondrial stress-associated endogenous MR1 ligands. The presence of such ligands can be assessed through a mass spectrometry analysis of VZV infected cell lysates, and cross referencing with the previously described MR1 binding nucleobase adducts (Vacchini et al., 2024).

From several studies published by our lab so far, it is clear that targeting the MR1 expression pathway is a conserved strategy encoded by several human herpesviruses (Ashley et al., 2023, McSharry et al., 2020, Purohit et al., 2021, Samer et al., 2024b). We orginally hypothesised that this strategy served as a counter measure for limiting riboflavin dependent activation of MAIT cells within a co-localised setting of intruding virus and resident microbiota. This hypothesis is now further complemented by increasing reports that MR1 can also present cellular stress derived ligands; akin to those potentially also generated during viral infection. Ultimately, as our understanding of the MR1 antigen repertoire grows, in parallel so does the list of evolutionary imperatives driving viral suppression of the MR1 antigen presentation pathway.

5.3 VZV infection of MAIT cells (Chapter 3)

The ability for VZV to infect T cells and utilise their migratory capacity for host-wide dissemination is now well-established (Ku et al., 2002, Ku et al., 2004, Moffat et al., 1995, Moffat et al., 1998). More recently, our lab has also described the capacity for VZV to infect and upregulate the skin homing capacity of circulating NK cells

(Campbell et al., 2018). At the time of our investigation, there was a paucity of information regarding how VZV interacts with unconventional innate-adaptive immune populations. In the third chapter of this thesis, we discovered that VZV infects circulating MAIT cells at an efficiency that was comparable to it's conventional T cell counterparts ($CD4^+$ and $CD8^+$ T cells). We found that VZV infected MAIT cells displayed a higher expression of CD69 and CD71, whilst maintaining the endogenously high skin homing program. Finally, the infection of MAIT cells was revealed to be productive, as infected MAIT cells transferred replicative virus to healthy epithelial cells.

Whilst assessing infection rate of MAIT cells across a time-course of 6 hours to 72 hours post VZV inoculation, we found that infection rates did not significantly increase beyond the first 24 hours. This was also previously observed in VZV co-cultured NK cells, whereby the level of VZV infection did not significantly increase after 6 hours post-inoculation (Campbell et al., 2018). These findings suggest a potential subpopulation within each lymphocyte compartment which is susceptible to VZV infection, as also previously hypothesised (Campbell et al., 2018). Previous studies analysing circulating MAIT cells have demonstrated the presence of phenotypically and functionally heterogenous subpopulations (Dias et al., 2018, Dias et al., 2017), therefore suggesting the potential for biased VZV infection in distinct subsets. This hypothesis can be tested holistically using spectral cytometry to assess surface markers expressed by VZV infected MAIT cells compared to exposed MAIT cells.

It is important to functionally assess if the retained extravasation and skin homing program expressed by VZV infected MAIT cells generates an intact skin homing

capacity. This could be addressed through chemotaxis assays utilising cognate skin homing chemokines such as CCL17 and CCL22 (Yoshie and Matsushima, 2015). Furthermore, the extravasation ability of VZV infected MAIT cells can be assessed through flow chamber assays; as recently utilised to reveal the high extravasation potential of healthy circulating MAIT cells (Lee et al., 2018). Finally, given that MAIT cells represent up to 2% of T cells in healthy human skin (Provine and Klenerman., 2020), *in situ* examination of MAIT cell frequencies within varicella lesions through skin biopsies could reveal whether VZV infection drives skin infiltration of MAIT cells compared to healthy controls.

It is difficult to ascertain the *in vivo* contribution towards viral dissemination that infection of MAIT cells by VZV provides, given that VZV exclusively infects humans. However, we can glean information through observing infection studies of rhesus macaques with the closely related herpesvirus Simian Varicella Virus (SVV), which also recapitulates all three stages of pathogenesis: primary infection, latency and reactivation (Messaoudi et al., 2009). Indeed, a recent transcriptomic analysis of responses of lung immune cells of rhesus macaques to SVV infection *in vivo* reported preferential infection of lung residing MAIT cells (Doratt et al., 2024), therefore supporting our hypothesis that MAIT cell infection is potentially an important contributor to VZV pathogenesis.

Along with negating host anti-viral responses, viruses must also leverage the host cell's metabolic processes to fuel the bio-energetically expensive process of producing viral progeny (Moreno-Altamirano et al., 2019). This has been previously demonstrated across several types of viral infections such as Influenza which induces

glycolysis to facilitate replication (Ren et al., 2021). Whilst transcriptomic analysis of SVV infected macrophages and T cells have also revealed enhanced glycolytic and oxidative phosphorylation programs (Doratt et al., 2024). Additionally, previous studies investigating VZV pathogenesis have also reported preferential infection of mature T cells (memory T cells) and mature NK cells (Ku et al., 2002, Sen et al., 2014a, Campbell et al., 2018). Importantly, these differentiated mature subsets also possess greater metabolic capacities such as enhanced glycolysis and fatty acid oxidation (Dimeloe et al., 2016, O'Sullivan et al., 2014, Keating et al., 2016). Overall, these studies are in congruence with our findings from VZV infection of MAIT cells which also demonstrated an upregulation of CD69 and CD71; which denote early activation as well as metabolic activity (Cibrián and Sánchez-Madrid, 2017, Conde et al., 1996, Shipkova and Wieland, 2012, Lo, 2016). Thus, these findings in conjunction with prior literature, will underpin further characterisation of the metabolic adaptations required for optimal VZV infection of several lymphocyte populations, including MAIT cells.

The rapid tissue migrating capacity of MAIT cells is predominantly understood to confer the host with protection at barrier sites (Provine and Klenermanl., 2020). Here, we instead reveal how the same hard-wired tissue migration program of MAIT cells can instead be exploited by VZV to potentially aid in the host wide dissemination of infective virus.

5.4 Functional impairment of MAIT cells during VZV infection (Chapter 4)

Our prior observation of increased early activation marker (CD69) expression by VZV infected MAIT cells suggested that infection potentially results in immune activation (Purohit et al., 2023). Furthermore, there remains no other reports describing the

capacity for MAIT cell functionality following any direct viral infection. Therefore, in the fourth chapter of this thesis, we assessed the ability for MAIT cells to functionally respond to activation stimuli following VZV inoculation. We found that both VZV exposed (viral antigen negative) and VZV infected (viral antigen positive) MAIT cell populations demonstrated impairment of activation, cytokine expression and cytolytic capacity in response to TCR dependent and cytokine stimulation.

Despite an average of approximately 20% of MAIT cells indicating infection, the entire population of MAIT cells exposed to VZV displayed abrogated functional responses to stimulation. Furthermore, VZV exposed cells isolated and cultured for 24 and 48 hours remained VZV antigen negative, thus suggesting a bona-fide bystander population. In combination, these findings have a striking implication that direct infection by VZV is not a prerequisite for functional paralysis. Indeed, this bystander phenotype was also previously observed in VZV exposed but not infected NK cells (Campbell et al., 2019). It is therefore pertinent to further investigate the mechanistic basis driving this bystander effect across distinct lymphocyte subsets. A recent paper investigating the release of host derived small extracellular vesicles (SEVs) following VZV infection of primary neurons demonstrated that isolated SEVs could dampen host immune responses in the absence of direct viral infection (Niemeyer et al., 2024a). Furthermore, these isolated SEVs contained a VZV immediate early protein IE62; which plays several immune-suppressive roles such as blocking interferon regulatory factor (IRF) 3 activity (Sen et al., 2010). This raises the possibility that VZV could suppress immune functions in distal anatomical locations through widespread release of SEVs.

However, it is important to note that in our study and previous investigations, both the suppression of NK and MAIT cell activation in bystander cells was shown to be contact-dependent (Campbell et al., 2019, Purohit et al., 2024). Previous studies have also shown that VZV infected melanocytes exhibit morphological abnormalities such as actin cytoplasmic extensions which serve as “viral highways” to traffic replication deficient VZV particles (light particles) (Carpenter et al., 2008). Importantly, these light particles retain their envelope structure and tegument proteins whilst lacking capsid and viral DNA, and furthermore are produced at a rate of approximately 40,000 light particles to 1 heavy (replication capable) particle (Carpenter et al., 2009, Carpenter et al., 2008). This raises the intriguing possibility that VZV infection produces abundant decoy particles potentially capable of overwhelming neighbouring cells through direct contact. Therefore, whilst the findings from this thesis do not necessarily exclude the role of SEVs, they strongly indicate the requirement for contact-dependent release of either SEVs or replication deficient particles that suppress the immune capacity of adjacent uninfected but exposed cells.

Furthermore, previous reports have demonstrated that HSV-1 virion entry but not productive infection is sufficient to inactivate TCR signal transduction; therefore resulting in downregulation of IL-2 production (Sloan et al., 2006). Interestingly, rewiring of the TCR signalling cascade by VZV has been previously illuminated in tonsillar T cells (Sen et al., 2014a). Therefore, assessing the TCR signalling cascade of VZV exposed and infected MAIT cells through phosphorylated-flow cytometry would reveal whether VZV remodels TCR transduction pathways.

Finally, it remains to be shown if VZV can also suppress MAIT cell functionality in tissue residence sites such as skin, gut or liver. This would be pertinent as our investigations currently show VZV modulation of MAIT cells that were unstimulated at the time of inoculum co-culture. This would be in contrast to tissue-resident MAIT cells which may receive continual TCR signalling by commensal microbes (Hinks et al., 2019, Lamichhane et al., 2019, Leng et al., 2019, Constantinides et al., 2019, Legoux et al., 2020, Ito et al., 2024) as well as tissue maintenance signals such as IL-7 which is constitutively expressed by hepatocytes (Sawa et al., 2009). Importantly, homeostatic commensal derived TCR signalling is proposed to drive MAIT cell tissue repair functions rather than a pro-inflammatory response (Ito et al., 2024, Constantinides et al., 2019, Hinks et al., 2019, Leng et al., 2019, Du Halgouet et al., 2023). Therefore, both the ability of VZV to disrupt tissue repair functionality of MAIT cells as well as their ability to modulate pre-activated MAIT cells should be assessed. The latter could reveal strategies to mitigate VZV induced MAIT cell dysfunctionality, as previously shown with the ability of IL-7 to rescue MAIT cell functionality in the context of chronic HIV infection as well as SARS-COV-2 infection (Leeansyah et al., 2015b, Hubrack et al., 2021).

Overall, our work defined in the fourth chapter of this thesis remains as the only report so far to explore direct pathogenic modulation of MAIT cell functionality.

Final statement

The MR1-MAIT cell axis is a deeply conserved mammalian host immune response that is embedded across mucosal barriers and functions as a sentinel checkpoint of pathogen intrusion. Prior to the undertaking of this thesis, there was only one published report from our lab that described modulation of MR1 antigen presentation by herpesviruses; HSV-1, HCMV and murine CMV. The work from this thesis now adds to this germinal field and further illuminates a profound manipulation on the entire MR1-MAIT cell axis by VZV. Given that the MR1-MAIT cell axis plays a pivotal role against several pathogens, it is likely that subversion of this axis is an evolutionary convergent strategy employed by other significant human pathogens. Therefore, the findings from this thesis reveals a fundamental perspective shift and presents a promising foundation that predicates the exploration of how human pathogens can directly counteract this ancient line of host defence.

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