# University of Cincinnati

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**I, Shu-en Wu , hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Molecular Genetics, Biochemistry, & Microbiology.**

It is entitled:

**Molecular Studies of Host Pathogen Interactions in Human Cytomegalovirus Infected Myeloid Cells**

Student's name: **Shu-en Wu**

This work and its defense approved by:

**Committee chair: William Miller, Ph.D.**

**Committee member: Rhett Kovall, Ph.D.**

**Committee member: Carolyn Lutzko, Ph.D.**

**Committee member: Simon Newman, Ph.D.**

**Committee member: Richard Thompson, Ph.D.**

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# Molecular Studies of Host Pathogen Interactions in Human Cytomegalovirus Infected Myeloid Cells

A dissertation submitted to the
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Shu-en Wu
B.S. National Chung-Hsing University, 2002
M.S. National Yang-Ming University, 2004

Committee Chair: William E. Miller, Ph. D.

**Abstract**

Human cytomegalovirus (HCMV) is a member of the subfamily of *Beta-herpesviridae* that establishes latency in human hematopoietic progenitor cells, myeloid progenitor cells and monocytes. While the serological positive population is prevalent worldwide, this virus does not cause severe symptoms in immunocompetent individuals. However, in immunocompromised patients and developing fetuses, this virus can cause serious morbidity and mortality. In light of many chronic diseases in the modern world leading to immunodeficiency and organ transplants, the importance of HCMV in medical research and public health is rising. Although it is known that HCMV establishes latency after primary infection, the molecular mechanism(s) that controls latent/lytic switch remains elusive. In addition, there is limited knowledge about stimuli that could induce the myeloid cells to support HCMV lytic phase.

In this thesis, we studied the effect of vitamin D, a hormone produced by human body or acquired from diet, on HCMV replication in peripheral monocytes and THP-1 cells. We found that vitamin D induces monocyte differentiation to facilitate HCMV replication, and instead of causing a direct up-regulation of HCMV immediate-early gene promoter (MIEP) activity as phorbol 12-myristate 13-acetate (PMA) treatment, the vitamin D induced monocyte differentiation causes an open chromatin structure around MIEP enhancer region of HCMV genome which also happens in PMA treated

monocytes.

In Chapter III of the thesis, we examined the Gαq dependent signaling pathways triggered by herpesviruses encoded G protein-coupled receptors by using Gαq specific inhibitor (YM-254890). We found that the inositol triphosphate accumulation triggered by US28 and M33 can be blocked by the inhibitor but the inositol triphosphate accumulation initiated by ORF74 cannot be suppressed by the inhibitor. In addition, CREB and NFAT activated by US28 and M33 can also be inhibited by the inhibitor, showing that they are Gαq dependent. However, for both US28 and M33, NF-κB is Gαq independent. Although ORF74 activates the same downstream signaling molecules including CREB, NFAT, and NF-κB, none of these is activated by Gαq dependent signal.

Finally, in Chapter IV of this thesis, we study the expression and function of US28 in THP-1 cells and monocytes. Our data showed that US28 protein is expressed in infected THP-1 cells, and that ectopic expression of US28 kindles Gαq dependent inositol triphosphate accumulation in THP-1 cells. In addition, the US28 triggered constitutive G$\alpha$q signal negatively regulates random migration and chemotaxis, and promotes THP-1 cells to adhere to endothelial cells though G$\alpha$q-PLC$\beta$-PKC signal axis.

## Dedication

This work is dedicated to the loving and cherished memories of my both grandmothers Nan-zhen Zhu, and Fu-chen Wu, and my twenty year old dog, Xiao-fu Wu.

## Acknowledgments

I would like to express my sincere gratitude to my advisor Dr. William E. Miller. Throughout my time in Ph. D. program, his enthusiasm for science also galvanizes my scientific interests, and he is always willing to listen to some of my whimsical scientific thoughts and to discuss with me. I also very appreciate that Dr. Miller always allow me to have freedom to try many experiments that I am interested in. It really helps me to sharpen my thoughts and skills in science. In addition, Dr. Miller is a very patient mentor. During the time in my graduate study, there was a period of time in which my research progression was stagnant but he still encouraged me and trusted me. Besides, to read article written by a foreigner like me does ask for a lot of patience and imagination to understand it, and Dr. Miller spent time reading through and editing my writings. Moreover, Dr. Miller is also a mentor who is kind to and cares about his students and lab members.

Special thanks are also due to my committee members Dr. Carolyn Lutzko, Dr.

Richard Thompson, Dr. Rhett Kovall, and Dr. Simon Newman. All the discussions we had in the committee meetings were very helpful, and the pSlik-Venus vector that Dr. Lutzko generously gave to us turned out to be an especially useful tool, and indeed helps us to make a breakthrough in the research of US28. Dr. Richard Thompson and his research assistant Tara Riddle often provided me some reagents, competent cells, or equipment for testing experiment conditions or trouble shooting. I would also like to thank Dr. Michael Lieberman, who allowed me to be his teaching assistant in the biochemistry class that offered me great opportunity to review the biochemistry knowledge and to obtain teaching experience. His lecture in the class is definitely the best biochemistry class I have attended.

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Finally, I would like to thank my parents, Chin-bang Wu, Tai-ling Lee, and my sister Shih-en Wu for their encouragement, their prayers, and their patience for listening to some of my whining about frustrations. Their supports indeed give me the courage to

keep going. My twenty-year dog, Xiao-fu, who brought me so many lovely memories during these years, is always my best buddy and loyal companion. I really thank God for His providence and His mercy to me. His words in Bible do give me peace, wisdom and strength to help me grow.

## Table of Contents

## List of Figures

**Chapter IV.**

## Abbreviation and Symbols

| | |
|---|---|
| ADP | adenosine diphosphate |
| BCL-6 | B-cell lymphoma 6 protein |
| CREB | cAMP response element binding protein |
| CMV | cytomegalovirus |
| DAG | diacyglycerol |
| DMEM | Dulbecco's modified essential medium |
| GDP | guanosine diphosphate |
| GPCR | G protein coupled receptor |
| GTP | guanosine triphosphate |
| HCMV | human cytomegalovirus |
| $IP_3$ | inositiol 1,4,5-triphosphate |
| KO | knockout |
| KSHV | Kaposi's sarcoma herpesvirus |
| MCP-1 | monocyte chemotactic protein 1 |
| MEF | mouse embryonic fibroblast |
| MEM | minimal essential medium |
| NF-κB | nuclear factor-kappa B |
| ORF | open reading frame |
| PKC | protein kinase C |
| PLC | phospholipase C |
| RANTES | regulated upon activation of normal T-cells expressed and secreted |
| RPMI-1640 | Roswell Park Memorial Institute -1640 |
| VDR | vitamin D receptor |
| vGPCR | viral encoded G protein coupled receptor |
| WT | wild type |
| α | alpha |
| β | beta |
| Ci | Curi |
| °C | Celsius degree |
| g | gram |
| l | liter |
| μ | micro |
| m | milli |
| M | molar |
| n | nano |
| xg | G force |

# Chapter I

# Introduction

**Introduction to human cytomegalovirus, pathogenesis, and disease**

Human cytomegalovirus (HCMV) is a β-herpesvirus that can infect a broad spectrum of cell types(1), and about 50-70% of worldwide population is serologically positive for HCMV(2,3). HCMV is an enveloped double stranded DNA virus with a genome size of 235 kilobase (kb) pairs(4). While in immunocompetent individuals HCMV infection does not cause severe symptoms(5), in immunocompromised patients HCMV can cause devastating symptoms such as pneumonia, retinitis and chronic graft rejection(6-8).

HCMV primary infection is also a leading cause of congenital defects. In the United States, about 1 in 150 children are born with congenital HCMV infection, and about 1 in 5 of these children born with congenital HCMV infection will have permanent medical sequelae(9-11). Although HCMV was first discovered over six decades ago (12,13), no vaccine for this virus is available (14,15). The development of an anti-CMV vaccine remains the highest priority according to the Institute of Medicine at the National Academy of Science(16). While drugs such as ganciclovir and valganciclovir are commonly used to treat HCMV infection, these drugs can lead to serious side effects, and the appearance of drug resistant viral strains is common (17). Therefore, more detailed studies of HCMV gene products and their effects on viral pathogenesis are critical if we are going to develop improved treatments for this

virus.

Similar to the other herpesviruses such as Herpes Simplex Virus and Epstein-Barr Virus, HCMV can establish latency after primary infection(18). The reservoir for HCMV latency is hematopoietic and myeloid progenitor cells and monocytes(19). Since no infectious virus is produced during the latent phase and viral reactivation can lead to devastating HCMV disease in immunocompromised individuals(20), it is essential to understand the mechanisms that the virus uses to regulate latency and targeting the lytic switch could be a viable approach for the generation of new therapies that could intervene with the virus production. Although some viral and cellular factors are known to be involved in the control of the latent/lytic

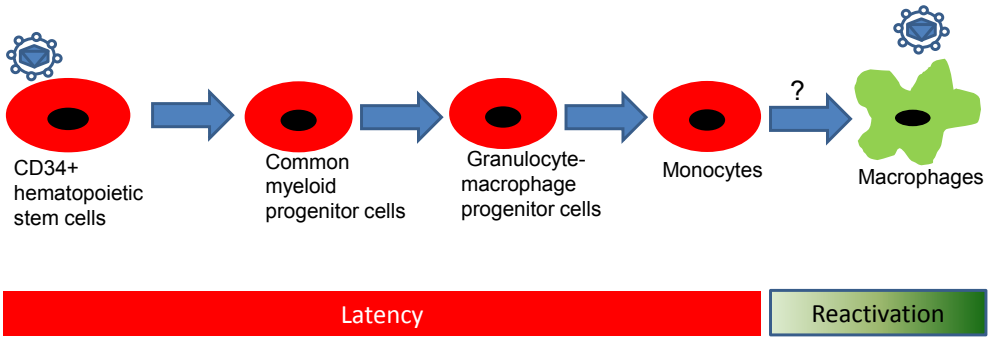


**Figure 1. Model depicting human cytomegalovirus (HCMV) latent and lytic phases in myeloid cells.**

HCMV enters the latent phase after infecting hematopoietic stem cells, common myeloid progenitor cells, granulocyte-macrophage progenitor cells, and monocytes. When monocytes differentiate into macrophages or mature dendritic cells, HCMV commonly reactivates. Macrophages and mature dendritic cells infected de novo with HCMV also support permissive infection. The molecular mechanisms that trigger HCMV reactivation and/or allow permissive infection in macrophages and mature dendritic cells are still not fully understood.

switch (21-23), the exact mechanisms remain unresolved. Moreover, while allogeneic stimulation has been shown to trigger HCMV reactivation(24), the complete repertoire of stimuli that can initiate viral reactivation and prime cells to support permissive infection remains unknown (Figure 1).

**Hypotheses and aims of the thesis**

In this research, we hypothesized that there could be unidentified physiological factors present in circulatory system that modulate latent/lytic phase switching in HCMV infected myeloid cells. Moreover, we speculated that YM-254890, a G$\alpha$q/11 specific inhibitor (25), could be used to dissect signals initiated by herpesvirus encoded G protein-coupled receptors (GPCRs). Lastly, based on previous research showing that the HCMV encoded GPCR US28 is transcribed during latency (26-28), we hypothesized that US28 protein is expressed during viral latency, and that the signals triggered by US28 could alter the biology of monocytes to facilitate viral dissemination.

Based on our stated hypotheses, we explored the following three specific aims. The first aim of this thesis is to identify additional factors that can facilitate HCMV lytic phase replication in myeloid cells. The identification of such factors will give insight into the mechanisms that facilitate the latent/lytic switch and also provide an appropriate platform to examine the functional activity of HCMV gene products such

as the viral G-protein coupled receptors (vGPCRs) in infected myeloid cells.

1,25-dihydroxyvitamin D3 (Vitamin D) is a natural hormone produced by the human body, which can also be obtained from diet (29-31). While vitamin D has long been appreciated for its role in regulating the homeostasis of calcium and phosphorus(32,33), a variety of more recent studies have also shown that vitamin D can regulate both innate and adaptive immunity and promote monocyte-macrophage differentiation(34-36), thus facilitating anti-bacterial and anti-viral responses(37-39). While Vitamin D action can certainly be thought of as anti-microbial under some circumstances, the differentiation of myeloid cells is an important factor for the initiation of HCMV permissive infection and thus vitamin D may actually favor lytic HCV replication. Since there is no precedent for the effect of vitamin D on HCMV replication in myeloid cells, one of the goals of the thesis is to examine this question. In this study, we used peripheral blood monocytes and THP-1 cells, a promonocytic cell line(40), to investigate the effect of vitamin D. While phorbol 12-myristate 13-acetate (PMA), a synthetic molecule that resembles diacylglycerol by activating protein kinase C(41), can differentiate THP-1 cells into macrophage-like cells and support HCMV lytic phase(42), natural stimuli that can trigger HCMV lytic infection in primary monocytes and THP-1 cells is of major interest. Since the sTHP-1 cell is commonly used as a myeloid model for HCMV studies (43,44), grows easily in tissue

culture, and is amenable to genetic manipulation/gene knockdown approaches(45,46), it would be interesting to determine whether Vitamin D can drive HCMV lytic permissive infection in both primary monocytes and in THP-1 cells. Moreover, since Vitamin D and TGF-β synergize to enhance myeloid cell differentiation (47-49), it is possible that Vitamin D combined with TGF-β could have an additional influence on HCMV replication in myeloid cells. Finally, to determine whether vitamin D can affect cytomegalovirus replication *in vivo*, wildtype and $VDR^{-/-}$ Balb/c mice were infected with MCMV (strain pARK25/K181), and spleens were analyzed for viral replication and pathology at 4 days post-infection.

The second aim of the thesis is to use the Gαq specific inhibitor YM254890 (25) to dissect the signaling pathways triggered by viral G protein coupled receptors (vGPCRs) encoded by herpesviruses including human cytomegalovirus (HCMV), murine cytomegalovirus (MCMV) and Kaposi's Sarcoma-associated herpesvirus (KSHV). Previous studies have demonstrated that these vGPCRs are similar in structure to mammalian chemokine receptors (50), while they have the ability to bind to multiple chemokines. Unlike chemokine receptors that trigger signal transduction only after binding to ligands, these herpesvirus vGPCRs can trigger ligand independent signals (51). ORF74 is a GPCR encoded by KSHV (52) that may play a role in viral reactivation (53) and has oncogenic properties in that it is a strong inducer

of angiogenesis(54,55). US28, a HCMV encoded vGPCR(56), has been shown to promote smooth muscle cell migration(57,58) and function as a chemokine sink which may play a role in evasion of immune response(59). The MCMV encoded M33 has been known to profoundly affect viral growth in salivary glands of persistently infected mice (60).

Given the association of the herpesviruses and their encoded vGPCRs with a variety of disease processes (50,51), the identification of small molecules that can target the vGPCRs and inhibit their signaling could be candidates for next generation antivirals(61,62). It is known that these three vGPCRs can all constitutively activate phospholipase C and cause accumulation of the second messenger $IP_3$ accumulation(63-65). Moreover these vGPCRs share the ability to activate a similar repertoire of downstream signaling molecules including the CREB, NFAT, and NF-κB transcription factors (63,66,67). However, whether CREB, NFAT, and NF-κB are all activated through Gαq is unknown. Whether or not the activation of these transcription factors contributes to the biological activities of the vGPCRs is also unknown. Therefore, we used the Gαq specific inhibitor YM-254890 to examine which downstream signaling molecule are Gαq dependent. While studies have shown that vGPCRs are somewhat promiscuous in coupling to Gα subunits in a context dependent fashion (65,68,69), our studies with the YM-254890 compound will provide

important clarity regarding the signaling mechanisms used by the vGPCRs. Moreover, as some Gα subunits show tissue specific expression patterns (70,71) inhibitors like YM-254890 could be used to determine whether the signaling triggered by the vGPCRs in different cell types utilizes the same or different G-protein. Thus, the characterization of the activities of the YM-254890 compound towards the vGPCRs could prove to be a quick and valuable reagent that could be used to determine the mechanisms of vGPCR signaling in different cell types.

The Third aim of the thesis is to examine expression, signaling, and functional effects of US28 in myeloid cells. Our studies will entail the use of both primary monocytes and THP-1 cell line where appropriate. According to previous studies, it is known that US28 transcripts can be detected in latently infected THP-1 cells, hematopoietic progenitor cells, and granulocyte-macrophage progenitor cells (26-28,72). However, whether US28 protein is expressed in these cells and what function(s) US28 possesses in these cells remains unknown. We used an HCMV TB-40E mcherry strain that encodes a US28-FLAG fusion protein (64,73) to infect THP-1 cells, and took advantage of high-affinity anti-FLAG reagents to purify US28 protein from infected cells and confirmed its identity by western blot. HCMV TB-40E strain is a strain that demonstrates enhanced tropism for endothelial and myeloid cells in comparison to the lab adapted strains such as AD169 and Towne (74,75). Unlike

the AD169 and Towne strains which have multiple deletions in the genome, TB-40E strain contains an intact ULb' region, a genomic segment that is essential for efficient entry into endothelial and myeloid cells and latency establishment in the latter (74,76,77).

To study the function and signaling of US28 in THP-1 cells, we used lentivirus to deliver tetracycline inducible lentiviral vectors expressing US28 wildtype or US28 mutants in THP-1 cells. While US28 triggers constitutive Gαq-dependent signals in various cell types, whether US28 signals similarly in the myeloid setting remains unknown. Inositol phosphate ($IP_3$) accumulation assays will be executed in combination with pharmacological inhibitors (YM-254890, etc) to study the signals generated by US28 in THP-1 cells. Chemokine receptors (to which US28 is most similar) are known to regulate cellular migration(78), and since US28 has been shown to promote cell migration in vascular smooth muscle cells and macrophages(58,79) it will be interesting to examine the effects of US28 on monocytes in our system. We used standard Boyden-Chamber based transwell migration assays to examine the effect of US28 on both chemokinesis (spontaneous cellular movement) and chemotaxis (ligand directed cellular movement). Monocytes are believed to play an important role in HCMV dissemination by adhering to endothelial cells and extravasating into surrounding tissue, where they can differentiate into macrophages

and initiate viral reactivation (80,81). Thereby, it is possible that US28 could influence monocyte-endothelial adhesion. Monocyte-endothelial adhesion assays were performed to determine the effect of US28 on monocyte adhesion.

In summary, focusing on the myeloid setting, we have studied the effects of Vitamin D on HCMV lytic replication and examined the signaling and functional activities of the HCMV vGPCR US28. We have also characterized and validated the effects of the pharmacological inhibitor YM-254890 on herpesvirus vGPCR signaling and incorporated the use of this compound into our studies on US28 function in myeloid cells. We hope that the results from these studies will offer insight into HCMV pathogenesis and provide useful information regarding our understanding of the HCMV latent/lytic switch and the signaling pathways triggered by viral GPCRs. Finally, we hope that our studies provide an appropriate framework from which to begin to think about using novel compounds like YM-254890 to influence viral replication and possibly decrease the frequency and extent of HCMV associated disease.

# Chapter II

# The Human Cytomegalovirus Lytic Cycle Is Induced by 1,25-Dihydroxyvitamin D3 in Peripheral Blood Monocytes and in the THP-1 Monocytic Cell Line

Shu-en Wu and William E. Miller

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**Abstract**

Human cytomegalovirus (HCMV) resides in a latent form in hematopoietic progenitors and undifferentiated cells within the myeloid lineage. Maturation and differentiation along the myeloid lineage triggers lytic replication. Here, we used peripheral blood monocytes and the monocytic cell line THP-1 to investigate the effects of 1,25-dihydroxyvitamin D3 on HCMV replication. Interestingly, 1,25-dihydroxyvitamin D3 induces lytic replication marked by up-regulation of HCMV gene expression and production of infectious virus. Moreover, we demonstrate that the effects of 1,25-dihydroxyvitamin D3 correlate with maturation/differentiation of the monocytes and not by directly stimulating the MIEP. These results are somewhat surprising as 1,25-dihydroxyvitamin D3 typically boosts immunity to bacteria and viruses rather than driving the infectious life cycle as it does for HCMV. Defining the signaling pathways kindled by 1,25-dihydroxyvitamin D3 will lead to a better understanding of the underlying molecular mechanisms that determine the fate of HCMV once it infects cells in the myeloid lineage.

## Introduction

Human cytomegalovirus (HCMV) is a β-herpesvirus that spreads broadly throughout the human population (1). In general, about 50-70% of people are serologically positive for HCMV worldwide (3). Although in immunocompetent individuals HCMV infection is typically asymptomatic, in the case of congenital infection, the virus can cause severe neurological sequelae such as deafness and developmental defects following infection of the fetus (9,10). In immunocompromised individuals, including those with HIV/AIDS or those receiving organ transplants, HCMV can cause devastating morbidity and mortality including pneumonia, retinitis, and transplant rejection (6-8). Moreover, many studies have shown that HCMV can be associated with chronic diseases such as atherosclerosis and hypertension, cancer, and autoimmune disease (82-85). Therefore, understanding the biology of HCMV infection is both clinically relevant and intensively studied with regards to potential pharmacological intervention. Like other herpesviruses, HCMV can establish latency in the human body, thus making the eradication of the virus from infected individual a difficult task (18,86). The cellular reservoirs for HCMV latency include hematopoietic stem cells, common myeloid progenitor cells, and monocytes (72,87-89). Deciphering the mechanisms that regulate the latent/lytic switch in HCMV infected cells could lead to the identification of novel therapeutics that could be used to regulate latency.

Previous studies have indicated that both viral and cellular factors are involved in the control of latent and lytic cycles in myeloid progenitors and monocytes (21-23,80,90-92); however, the molecular mechanisms remain unresolved, and it is highly probable that there are numerous cellular and viral regulatory factors that have yet to be identified. In light of this, further investigation of the mechanisms and factors that influence the switch between HCMV latency and lytic replication in clinically relevant myeloid cell types is needed.

It is known that the developmental maturation of monocytes into macrophages and dendritic cells can reactivate HCMV from latency leading to the production of new infectious virus (24,80,92-95). In addition, there are various extracellular stimuli (i.e. PMA) that can trigger monocyte to macrophage differentiation (96-101) and some of these stimuli have also been shown to directly induce the HCMV immediate-early gene promoter, which is essential for induction of the HCMV lytic cycle (102-104). Since the activities of these stimuli appear to be multi-factorial, it is difficult to determine if the major influence of these stimuli on lytic replication is induction of the IE promoter, promotion of cellular maturation/differentiation or a combination of both activities. THP-1 cells are a monocytic cell line that is commonly used in combination with primary blood derived monocytes to study the interaction between HCMV and myeloid cells and gain insight into the latent/lytic switch (44,105). It is well known that

HCMV enters latency or a quiescent state in undifferentiated THP-1, and the virus typically enters into the lytic cycle after it infects phorbol 12-myristate 13-acetate (PMA) treated THP-1 cells (42,106). As a consequence, PMA is a reagent of choice used to promote myeloid differentiation in studies aimed at inducing lytic replication in *in vitro* systems. However, PMA is a synthetic compound resembling diacylglycerol (DAG) that is capable of activating a broad range of cell signaling pathways (41,107,108). In this research we sought to identify additional physiologically relevant compounds that could trigger both monocyte differentiation and HCMV lytic infection. Vitamin D3 is a hormone that is produced by the human body and acquired in a supplemental fashion through diet (29-31). The most well-known effects of vitamin D3 and its active metabolite 1,25-dihydroxyvitamin D3 are to regulate homeostasis of calcium and phosphorus and promote bone development through interaction with the vitamin D receptor (VDR), a member of the nuclear receptor family of transcription factors (32,33). Interestingly, blood leukocytes robustly express the VDR and results of studies performed *in vitro* in human myeloid cell lines and *ex vivo* in murine bone marrow cells have demonstrated that 1,25-dihydroxyvitamin D3 has the ability to induce monocyte-macrophage differentiation (34,35,109-113). It is therefore not surprising that 1,25-dihydroxyvitamin D3 has been demonstrated to exhibit antibacterial and antiviral effects (37-39,114). The importance of

1,25-dihydroxyvitamin D3 in regulation of immune system function has been further highlighted by studies which suggest that 1,25-dihydroxyvitamin D3 or synthetic analogues of 1,25-dihydroxyvitamin D3 could be used as potent candidates for the treatment for autoimmune diseases, infectious diseases and anticancer therapies (115-117). Nonetheless, the effect of 1,25-dihydroxyvitamin D3 on HCMV replication in monocytes and macrophages remains unknown. Therefore, we explored the possibility that peripheral blood monocytes and THP-1 cells could be used to determine the effect of 1,25-dihydroxyvitamin D3 on HCMV replication in myeloid cells. According to the results of previous studies, 1,25-dihydroxyvitamin D3 treatment induces THP-1 cells to differentiate into mature monocytes, with high CD14 expression (34,118,119) and therefore we hypothesized that we also could use this model to study HCMV replication in 1,25-dihydroxyvitamin D3 treated cells that are in the transition from the promonocytic to macrophage stages.

Interestingly, we found that the HCMV lytic phase can be induced in 1,25-dihydroxyvitamin D3 treated primary monocytes and in THP-1 cells with infectious virus being produced by these cells. In contrast to PMA treated cells, 1,25-dihydroxyvitamin D3 does not have a direct effect on the HCMV immediate-early gene promoter in reporter gene assays suggesting that the predominant effect of 1,25-dihydroxyvitamin D3 is to drive differentiation and not necessarily to directly

stimulate IE promoter activity. When 1,25-dihydroxyvitamin D3 is combined with PMA to differentiate THP-1 cells, no additive effect on HCMV replication is observed. These results demonstrate that 1,25-dihydroxyvitamin D3 induces a set of differentiation related signaling pathways that creates a favorable cellular milieu for HCMV lytic infection. Moreover, our results suggest that clinical/dietary supplementation with vitamin D3 could be problematic in patients susceptible to reactivation-based HCMV disease.

**Materials and Methods**

**General Reagents.** 1,25-dihydroxyvitamin D3 and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich. APC conjugated anti-human CD14, anti-human CD11b, anti-human CD54, anti-human CD36 antibodies and PE conjugated anti-mouse IgG1 antibodies were obtained from eBioscience. Anti-CMV IE1/IE2 antibody (mAb810) and an Alexa Fluor® 488 conjugated version of mAB810 were purchased from Millipore. Anti-CMV UL44 antibody (mAb 25G11, IgG1 isotype) was a kind gift of John Shanley, and anti-CMV pp65 antibody was obtained from Virusys Corporation.

**Cell culture and differentiation of THP-1 cells.** THP-1 cells were maintained in RPMI-1640 (Roswell Park Memorial Institute-1640) supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% $CO_2$. THP-1 cells were passaged every 3 days to maintain the cell density between $0.2x10^6$ and $1x10^6$ cells/ml. Human foreskin fibroblasts (HFFs) were maintained in DMEM (Dulbecco Modified Eagle's Medium) supplemented with 10% Fetal Clone III serum, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% $CO_2$. THP-1 cells were treated with 80nM PMA or 100nM 1,25-dihydroxyvitamin D3 for three days to induce cellular maturation/differentiation.

**Propagation and purification of virus.** The HCMV TB40E-mCherry(3XFLAGUS28) virus was generously provided by Dr. Christine O' Connor from the Cleveland Clinic (64,73). This virus was characterized and demonstrated to grow with similar kinetics and to similar titers as does HCMV TB40E. To propagate virus, HFFs were infected with TB40E viruses at an m.o.i. of 0.04. Viral supernatant was harvested at days 9, 11, and 13 post-infection. Cell Culture supernatant containing virus was centrifuged at 1800xg for 3 minutes at 21°C to remove cellular debris. The clarified supernatant was overlayed on a 20% D-sorbitol/1mM $MgCl_2$ cushion and subjected to ultracentrifugation at 24,000 rpm for 1hr at 21°C. Supernatant was decanted, and the

viral pellet was resuspended in RPMI-1640 culture media. Viral supernatant was aliquoted and stored at - 80°C.

**Isolation of monocytes from peripheral blood of normal donors.** Blood was diluted 2 fold with Dulbecco's Phosphate-Buffered Saline (DPBS) containing 2 mM EDTA. Diluted blood was carefully layered over 15 ml of Ficoll-paque® PLUS in a 50 ml conical tube. Conical tubes were centrifuged at 400xg for 30-40 minutes at 20°C in a swinging bucket rotor without brake. After centrifuge, the upper layer was aspirated, leaving buffy coat containing the mononuclear cell layer undisturbed at interphase. The buffy coat was transfered to a clean tube, and fresh DPBS with 2 mM EDTA was added to fill the tubes. The cells were centrifuged at 300xg for 10 minutes at 20°C. Then supernatant was carefully removed. In order to remove platelets, cells were resuspended in 50 ml of DPBS with 2 mM EDTA, and centrifuged at 200xg for 10-15 minutes at 20°C. The supernatant was removed afterward. The wash step was repeated once to further deplete platelets. Cells were resuspended in 80 µl of buffer (DPBS containing 0.5% BSA and 2 mM EDTA) per $10^7$ cells. 20 µl of CD14 MicroBeads (Miltenyi Biotec) was added per $10^7$ cells, and cells were incubated in the cold room (2-8°C) for 30 minutes. After incubation, cells were washed by adding 1-2 ml of buffer (DPBS containing 0.5% BSA and 2 mM EDTA) per $10^7$ cells, and

centrifuged at 300xg for 10 minutes. $10^8$ cells were resuspended in 500 µl of buffer, and passed through LS Column (Miltenyi Biotec). The column was washed 3 times with buffer. The column was removed from MACS Separator (Miltenyi Biotec), and cells were flushed out by firmly pushing the plunger into the column.

**Culture and infection of primary peripheral blood derived monocytes.** $CD14^+$ monocytes isolated as described above were resuspended in RPMI-1640 supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were then cultured in 1,25-dihydroxyvitamin D3 (100 nM), PMA (80 nM) or the appropriate vehicle control (EtOH or DMSO) for 2 days at 37°C in 5% $CO_2$. After 2 days of culture in 1,25-dihydroxyvitamin D3, PMA or vehicle, viral supernatant was added at a MOI of 10, and cells were incubated overnight. After overnight incubation with virus, cells treated with vehicle or 1,25-dihydroxyvitamin D3 were pelleted by centrifugation, viral inocula were removed, and cells were treated with 1X trypsin for 5 minutes to remove attached but un-internalized virions (120). Cells were then resuspended in fresh RPMI supplemented as described above and cultured for 4-6 days. In the case of the PMA treated cells that were adhered to the culture plates, viral inocula were aspirated, cells were washed thoroughly with DPBS, fed with fresh RPMI and supplements and cultured for 4-6 days.

**HCMV infection of THP-1 cells.** THP-1 cells were infected with HCMV TB40E-mCherry (3XFLAGUS28) at MOIs as indicated in the figure legends. After the viral supernatant was added, cells were centrifuged at 21°C and 1000xg for 30 minutes to enhance infectivity. After overnight culture, cells were spun down and the inocula were removed. Cells were incubated in 1X trypsin for 5 minutes to remove attached but un-internalized virions (120). The trypsin reactions were neutralized by adding equal volumes of fresh culture media. The supernatant is aspirated, and cells were resuspended in fresh culture media. Because the PMA-differentiated cells firmly adhere to the plates, the centrifuge step is omitted from infection protocol. After overnight culture, culture media were removed and cells were washed with 1X DPBS, and fresh media were added to the cells.

**Western blot analysis of HCMV gene expression.** $1x10^6$ infected THP-1 cells were lysed in NP-40 cell lysis buffer (50 mM HEPES pH7.4, 0.5% NP-40, 250 mM NaCl, 20% glycerol, 2 mM EDTA, 100 μM Sodium Orthovanadate, 1 mM Sodium Fluoride, 1X complete Mini protease inhibitor). Cell lysates were sonicated for 20 seconds on level 1 using a Sonic Dismembrator Model 100 (Fisher Scientific). Protein concentrations for each lysate sample were determined using the Bio-Rad protein assay reagent. Lysates were mixed with Laemmli sample buffer, and heated at 100°

for 10 minutes. 30 µg of protein was loaded into each lane for electrophoresis. Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Continental Lab Products). Membranes were blocked with 5% non-fat dried milk for 1 hour and membranes were incubated with primary antibody at 4° overnight. Membranes were washed 3 times with Tris buffered saline with Tween-20 (TBST) and then incubated with secondary antibody for 2 hours. Membranes were washed with TBST 3 times and subjected to antibody detection using the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). Luminescence emitted from the membranes was detected by classic blue autoradiography film BX. Films were developed by Kodak min-R mammography processor.

**MIEP reporter gene construction.** The HCMV immediate-early gene promoter (bp 52586-53162 in GenBank entry AC146907.1) was PCR amplified from FIX-BAC bacmid DNA using the following primers. HCMV MIEP promoter forward primer: 5'-TAACCCGGGTAGTAATCAATTACGGGG-3', HCMV MIEP promoter reverse primer: 5'-TCGAGATCTCTGACGGTTCACTAAACG-3'. PCR amplification was performed for 30 cycles, consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. The HCMV MIEP

promoter fragment was cloned into the XmaI and BglII sites of pGL3 and sequenced to confirm the identity of the MIEP fragment (Genewiz, Inc).

**Luciferase assays.** $2X10^5$ THP-1 cells were plated per well in 24-well plates and cultured overnight. For each transfection, 410 ng pcDNA3, 60 ng pGL3-HCMV MIEP, 30 ng phRGTK-renilla and 1.5 µl TransIT-2020 was diluted into 50 µl RPMI-1640 and incubated for 15 minutes. The incubated transfection reagent was then added into each well. 4 hours after transfection, vehicle (ethanol or DMSO), 1,25-dihydroxyvitamin D3 (100 nM), or PMA (80 nM) was added to the designated wells and the cells were incubated for a further 24 hours. Cells were lysed in 200 µl of 1X passive lysis buffer and 10 µl of the cell lysate was used in luciferase assay reactions. 50 µl of Firefly-luciferase substrate was added to cell lysate and luciferase activity was measured on a Glomax 20/20 luminometer (Promega). 50 µl Stop & Glow solution was added to each reaction, and luminescence of the control reporter Renilla-Luciferase was measured. The Firefly-luciferase reading of vehicle control (ethanol) was divided by the Renilla-Luciferase reading of vehicle control and that value was defined as 1. The fold changes were then determined by dividing the luciferase to renilla ratios of the experimental conditions to the ratio of vehicle control.

**Infectious center assays.** $1x10^5$ HFF cells were plated into wells of 12-well plates and cultured overnight. Infected THP-1 cells or primary monocytes were harvested at six days post-infection and co-cultured with HFFs for 2 days. Culture media was then removed and the plates were washed twice with 1X PBS. Cell monolayers were covered with overlay media (a 1:1 mixture of 1.5% carboxymethyl cellulose (Sigma), and 2X MEM supplemented with 20% FCIII serum, nonessential amino acids, and penicillin-streptomycin) and incubated for another 8 days to allow for plaque development. Cells were fixed with methanol, stained with 10% Geimsa (Sigma) and plaques were counted using a dissecting microscope.

**Plaque assays.** After infection, culture media from each cell sample was harvested on the indicated days, and 10 µl of culture media was added to HFF monolayers plated in 12-well plates the previous day. Virus was adsorbed to HFF monolayers for 3 hours, culture media were removed, and covered with overlay media (a 1:1 mixture of 1.5% carboxymethyl cellulose (Sigma), and 2X MEM supplemented with 20% FCIII serum, nonessential amino acids, and penicillin-streptomycin) and incubated for another 8 days to allow for plaque development. Cells were fixed with methanol, stained with 10% Giemsa (Sigma) and plaques were counted using a dissecting microscope.

**Flow cytometry.** For cell surface marker analyses, cells were harvested and resuspended in 50 µl of a 0.5% BSA/DPBS solution containing 1:200 dilution of the appropriate APC-conjugated antibody (CD11b, CD14, etc). Cells were incubated at room temperature for 1 hour. Cells were washed with 0.5% BSA/DPBS solution, resuspended in fresh DPBS and analyzed by flow cytometry on a BD FACSCalibur. For HCMV IE protein staining, cells were harvested, resuspended in 100 µl DPBS and 1 ml of 70% ice cold EtOH was added to fix the cells. Fixed cells were washed and permeabilized with 0.5% BSA/DPBS solution containing 0.5% tween-20. Cells were then resuspended in 0.5% BSA/DPBS solution containing 1:200 dilution of Alexa488 conjugated anti-HCMV IE antibody, and incubated at room temperature for 1 hour. Stained cells were washed with 500 µl 0.5% BSA/DPBS solution containing 0.5% tween-20. For UL44 and IE co-staining, cells were fixed and permeabilized as above and then resuspended in 0.5% BSA/DPBS solution containing 1:10 dilution anti-HCMV UL44 antibody. Cells were incubated for 1 hour at room temperature, washed as described above and then incubated for 1 hour in 0.5% BSA/DPBS solution containing 1:250 dilution of PE-conjugated anti-mouse IgG1 to label the UL44 primary antibody. UL44 stained cells were then washed and stained for IE proteins as described above. After staining, cells were resuspended in DPBS and analyzed by flow cytometry.

**Semi-quantitative PCR for viral DNA copy number.** 2x$10^5$ THP-1 cells were harvested on days 1 and 6 post-infection, and lysed in 100 µl DNA lysis buffer (121) containing 20 µg of Proteinase K at 55°C overnight. Proteinase K activity was stopped by incubating DNA lysates at 100°C for 15 minutes and DNAs were used for semi-quantitative PCR. Primers for HCMV IE amplification: HCMV IE forward 5'-ATGGAGTCCTCTGCCAAGAGAAAGATGGAC-3', HCMV IE reverse 5'-CAATACACTTCATCTCCTCGAAAGG -3' (122). Primers used for GAPDH amplification: GAPDH forward 5'-GAGCCAAAAGGGTCATC-3', GAPDH reverse primer 5'-GTGGTCATGAGTCCTTC-3' (123). PCR amplification was performed for 30 cycles, consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72° for 30 seconds. Band intensities were measured by NIH Image software and calculated as a ratio of HCMV IE DNA over cellular GAPDH DNA.

**Chromatin Immunoprecipitation.** 5X$10^6$ cells were harvested at 2 days post-infection and fixed in DPBS containing 1% formaldehyde for 10 minutes at room temperature. 2.5 M glycine was added to stop the fixation reaction, and cells were washed in ice cold DPBS. Cells were lysed in cell lysis buffer (0.5 mM PIPES pH 8, 85 mM KCl, 0.5% NP40, 1X protein inhibitor cocktail). Nuclei were pelleted by centrifuge

and resuspended in 500 µl nuclear lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS, 1X protein inhibitor cocktail). The lysates were sonicated for six cycles (30 seconds on/ 30 seconds off) using program 3 on a Sonic Dismembrator Model 100 (Fisher Scientific). Lysates were then diluted 5 fold in IP dilution buffer (0.01% SDS, 1.1% Triton-X 100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH8, 167 mM NaCl, 1X protein inhibitor cocktail) and pre-cleared with 30 µl sepharose beads for 2 hours at 4°C. 3 µg of anti-tri-methylated histone 3 antibody (Millipore) was added into each reaction and rotated overnight at 4°. Protein A/G beads were added to each reaction and incubated at 4°C for 3 hours to capture primary antibody. Protein A/G beads was pelleted by centrifugation and washed with 1X dialysis buffer (2 mM EDTA, 50 mM Tris-HCl pH8, 0.2% Sarkosyl, 1X protein inhibitor cocktail) 3 times. Bound chromatin fragments were eluted using elution buffer (50 mM $NaHCO_3$, 1% SDS). After elution, RNase A and NaCl were added to make final concentration of 0.083 mg/ml and 0.2 M respectively and the solution was incubated at 65°C overnight to reverse cross-links. 34 µg of Proteinase K was added to digest proteins at 45°C for 2 hours. Primers for amplification of MIEP enhancer: Forward primer: 5'-TTGGGCATACGCGATATCTG-3'. Reverse primer: 5'-GCCTCATATCGTCTGTCACC-3' (124). The DNA fragments were recovered using a Fermentus gel extraction kit and 20 ng of immunoprecipitated DNA was used for

PCR amplification. PCR reaction conditions are the same as mentioned above except were performed for 36 cycles. The signal from chromatin immunoprecipitation samples were normalized to signal from respective input samples.

**Statistical analysis.** Results are presented graphically and represent the mean ± S.E. of three or more experiments. The intensities of bands on immunoblots were quantified using Image J software. Statistical analyses were calculated with unpaired two-tailed Student's *t* tests using GraphPad Prism® 6 software. Differences were considered significant at $p < 0.05$. *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Nonsignificant changes are denoted by n.s., and represent $p > 0.05$.

## Results

### *1,25-dihydroxyvitamin D3 promotes HCMV replication in primary monocytes and THP-1 cells*

Vitamin D3 is a natural hormone that is produced by the human body and typically supplemented through diet (29-31). In addition to the regulation of the homeostasis of calcium and phosphorus (32,125), vitamin D3 has been shown to play multiple roles in immune responses including modulating T cell and B cell activity (126), promoting

monocyte-macrophage differentiation (36,127), stimulating the anti-bacterial and anti-viral effects of macrophages (128,129), and driving lineage commitment of hematopoietic progenitor cells (130). Vitamin D3 like other endocrine hormones is carried by the circulatory system to various tissues (29), and research has also shown that many cells within the immune system have the enzyme that can convert Vitamin D3 into its active form, 1,25-dihydroxyvitamin D3 (131-133). Therefore, vitamin D3 can execute its effects on a wide variety of cells in either an endocrine or paracrine fashion (134). HCMV is a β-herpesvirus which can infect myeloid cells and establish latent and/or lytic infections within cells of this lineage (19). Although studies have shown that allogeneic stimulation or stimulation with cytokines like TNF-α can stimulate HCMV IE promoter activity and drive lytic replication in myeloid cells (24,102,135), the effect of 1,25-dihydroxyvitamin D3 on HCMV replication in myeloid cells remains unexplored. To determine if 1,25-dihydroxyvitamin D3 may influence HCMV lytic replication in myeloid cells, we examined the effect of 1,25-dihydroxyvitamin D3 on the ability of CD14 positive peripheral blood monocytes to support lytic replication. Peripheral blood mononuclear cells (PBMCs) from healthy anonymous donors were isolated by Ficoll-paque and CD14 positive monocytes were subsequently isolated from PBMCs using CD14 magnetic beads (Miltenyi). Monocytes were treated with 1,25-dihydroxyvitamin D3 (100 nM) for 2 days and

infected with HCMV TB40E at an MOI of 10. On days 4 and 6 post-infection, cells were harvested and subsequently co-cultured with human foreskin fibroblasts (infectious center assays) to determine whether the infected monocytes were producing infectious virus. Interestingly, monocytes treated with 1,25-dihydroxyvitamin D3 exhibited a 5-8 fold increase in infectious centers over cells treated with the vehicle control ethanol (Figure 2A). These data indicate that 1,25-dihydroxyvitamin D3 treatment can create a milieu in blood monocytes that more efficiently supports HCMV virus production. HCMV replication in primary monocytes treated with the phorbol ester phorbal 12-myristate 13-acetate (PMA) was similarly examined (Figure 2B). PMA is a well-established inducer of HCMV replication in a number of systems (42,106). PMA treated cells exhibited a 15-30 fold increase in infectious centers over cells treated with the vehicle control DMSO (Figure 1B). Thus, for comparison, while it is readily apparent that 1,25-dihydroxyvitamin D3 is a robust inducer of HCMV replication in monocytes, the level of replication achieved is not as strong as that achieved by the phorbol ester, consistent with what might be expected for a natural compound like 1,25-dihydroxyvitamin D3.

We then sought a model system that could be utilized to provide a more mechanistic explanation for this finding. THP-1, an established monocytic cell line and model system frequently used in HCMV studies (43,44), was then deployed to further

explore the effects of 1,25-dihydroxyvitamin D3 on HCMV lytic replication. Since it is well established that PMA can drive HCMV production in THP-1 cells, we again used PMA as a control in these experiments (42). THP-1 cells were treated with vehicle (ethanol), 1,25-dihydroxyvitamin D3 (100 nM) or PMA (80 nM) for 3 days before infection. Cells were then infected with HCMV TB40E at an MOI of 10. On day 6 post-infection, THP-1 cells were co-cultured with HFFs in infectious center assays. In THP-1 cells treated with the ethanol control, only a very low number of plaques were detected in infectious center assays, which indicate that THP-1 cells rarely support lytic phase replication after HCMV infection (Figure 3). In PMA treated THP-1 cells, there was a 40-fold increase in the number of plaques arising in infectious center assays supporting early studies that reported the induction of lytic phase replication by PMA treatment. Importantly, 1,25-dihydroxyvitamin D3 treatment of THP-1 cells resulted in a 10-fold increase in the number of plaques arising in infectious center assays. We repeated this experiment and examined infectious center production at days 6 and 8 post-infection and obtained similar results, indicating that the difference in lytic replication observed between 1,25-dihydroxyvitamin D3 and PMA treated cells is not simply the result of a delay in virus replication in the 1,25-dihydroxyvitamin D3 treated cells (data not shown). Therefore, since 1,25-dihydroxyvitamin D3 can promote lytic virus production in both peripheral blood monocytes and in the

monocytic cell line THP-1, we conclude that THP-1 cells would provide a viable model to recapitulate and further explore the effects of 1,25-dihydroxyvitamin D3 on HCMV replication in myeloid cells.

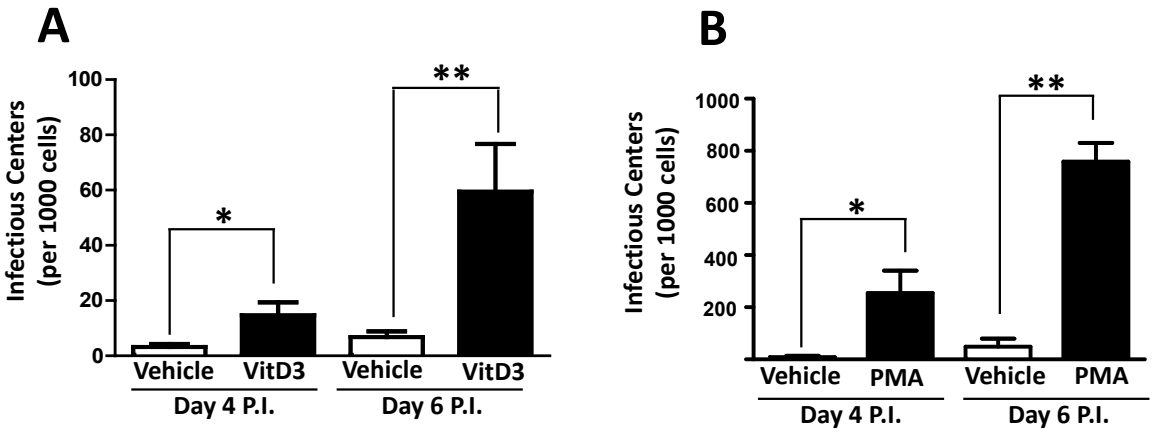



**Figure 2. 1,25-dihydroxyvitamin D3 promotes HCMV replication in primary peripheral blood derived monocytes**

Monocytes were treated with 100 nM 1,25-dihydroxyvitamin D3 (A) or 80 nM PMA (B) for 2 days and then infected with HCMV TB40E at a MOI of 10. On 4 days and 6 days post infection, cells were harvested and co-cultured with HFF fibroblasts for 2 days. After 2 days of co-culture, fibroblast monolayers were overlaid with CMC/MEM and incubated for 8 days to allow for plaque development. The data represent 4-8 independent experiments performed in duplicate. VitD3, 1,25-dihydoxyvitamin D3. * $p<0.05$, ** $p<0.01$.

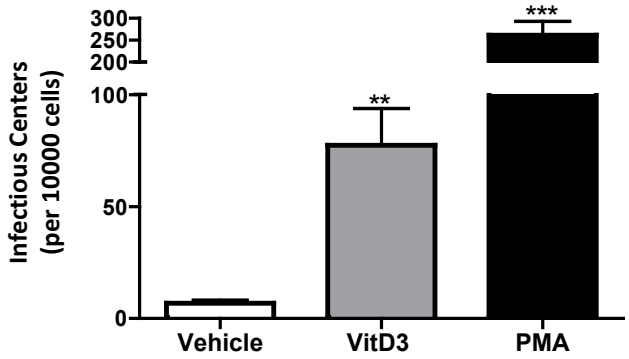



**Figure 3. 1,25-dihydroxyvitamin D3 promotes HCMV replication in the THP-1 monocytic cell line**

THP-1 monocytes were treated with 1,25-dihydroxyvitamin D3 (100 nM) or PMA (80 nM) for 3 days and then infected with HCMV TB40E at a MOI of 10. On day 6 post-infection, cells were harvested and co-cultured with HFF fibroblasts for 2 days. After 2 days of co-culture, fibroblast monolayers were overlaid with CMC/MEM and incubated for 8 days to allow for plaque development. The data represent five independent experiments performed in duplicate. VitD3, 1,25-dihydoxyvitamin D3. ** $p<0.01$, *** $p<0.001$.

***1,25-dihydroxyvitamin D3 treatment does not influence the ability of HCMV to establish an initial infection in monocytes***

Since we found that 1,25-dihydroxyvitamin D3 treatment can dramatically increase the number of plaques that arise from THP-1 cells in co-culture infectious center assays, we wanted to determine whether this difference could be the result of increased infectivity or entry of HCMV virions into 1,25-dihydroxyvitamin D3 treated cells. We used semi-quantitative PCR to examine viral genome copy number in cells at various time points post-infection (Figure 4). If 1,25-dihydroxyvitamin D3 leads to increased infectivity of the monocytes, we would expect to see increased viral DNA

levels at early time points post-infection. However, at day 1 post-infection, cells treated with 1,25-dihydroxyvitamin D3 or PMA exhibited similar viral copy numbers to that of vehicle control cells. The results are depicted in Figure 4A and quantitative results from six independent experiments are shown in Figure 4B. This result suggested that HCMV infects control and 1,25-dihydroxyvitamin D3 treated cells with equivalent efficiency. At day 6 post-infection, the PCR signal for viral genomes in control THP-1 cells declined while the signal from 1,25-dihydroxyvitamin D3- and PMA-treated cells was maintained or increased, consistent with the conclusion that 1,25-dihydroxyvitamin D3 and PMA treated cells are supporting lytic HCMV replication.

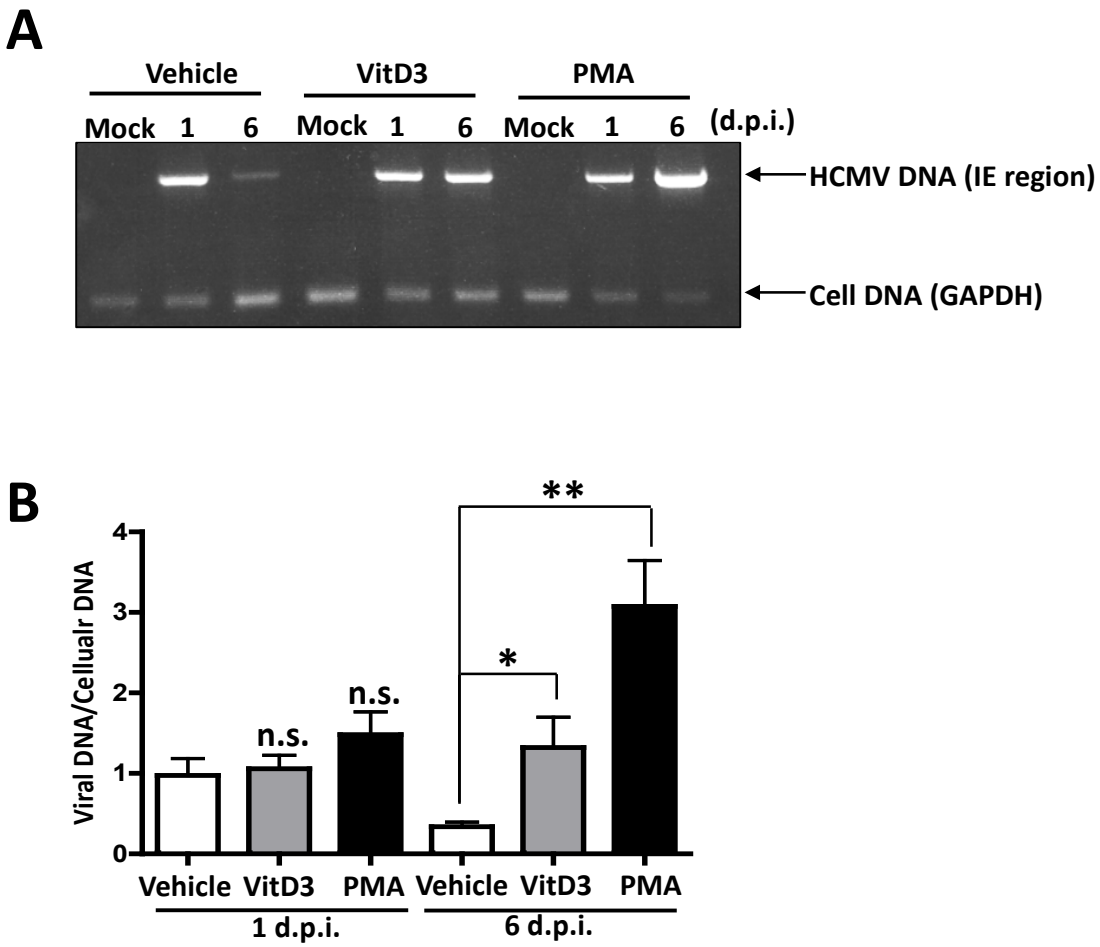



**Figure 4. 1,25-dihydroxyvitamin D3 treatment does not influence HCMV entry**

(A) THP-1 monocytes were treated with 1,25-dihydroxyvitamin D3 (100nM) or PMA (80nM) for 3 days and then infected with HCMV TB40E at a MOI of 10. On days 1 and 6 post infection, DNA from THP-1 cells subjected to the indicated treatments was amplified by PCR for HCMV genomes (IE region) or cellular genomes (GAPDH). PCR products were visualized by agarose gel electrophoresis. (B) The PCR signals of viral DNA were normalized to the signals of GAPDH. Data shown are the means +/- SEM of six independent experiments. VitD3, 1,25-dihydroxyvitamin D3. n.s. non-significant, *p<0.05, **p<0.01, d.p.i. day post infection.

***1,25-dihydroxyvitamin D3 treated THP-1 cells are more likely to exhibit IE gene expression following infection***

While an equivalent amount of HCMV DNA is initially present following infection of control or 1,25-dihydroxyvitamin D3 treated cells, it is clear that the 1,25-dihydroxyvitamin D3 treated cells support a robust increase in productive HCMV replication. Therefore, we next chose to examine HCMV gene expression profiles in 1,25-dihydroxyvitamin D3 and PMA treated cells. Immediate early (IE) gene expression is typically repressed in cells that fail to undergo lytic phase induction, but is expressed rapidly after infection in cells capable of supporting lytic replication (43,136,137). We examined IE gene expression in cells treated with vehicle control, 1,25-dihydroxyvitamin D3 or PMA (Figure 4). Flow cytometric staining with anti-IE-Alex488 antibodies was performed as this enabled us to assess not only the frequency with which IE positive cells arise but also the relative level of IE antigens per cell. Infected cells were harvested and examined at day 1 post-infection. Compared to vehicle control, 1,25-dihydroxyvitamin D3 treatment resulted in a significantly higher percentage of IE positive cells by day 1 post-infection (Figures 5A and B). Interestingly, while the number of IE positive cells is significantly increased with 1,25-dihydroxyvitamin D3 treatment, there is no difference in the relative IE expression per cell as the mean fluorescent intensities are similar when comparing

vehicle and 1,25-dihydroxyvitamin D3 treated cells (Figure 5C). PMA, in contrast, caused an increase in both the percentage of IE positive cells and relative IE expression per cell, suggesting that the mechanisms utilized by 1,25-dihydroxyvitamin D3 and PMA to promote lytic replication may be distinct. We did not detect any differences in the subcellular localization of IE1/2 when comparing control, 1,25-dihydroxyvitamin D3, and PMA treated cells indicating that changes in the compartmentalization of IE proteins is unlikely to account for the mechanism of 1,25-dihydroxyvitamin D3 induced HCMV replication (data not shown).

Taken together, while viral genome copy numbers are initially equivalent, the 1,25-dihydroxyvitamin D3 treated cells are more highly likely to be capable of initiating IE protein production consistent with their ability to progress to the lytic phase (Figures 3 and 5). Moreover, while 20-40% of THP-1 cells treated with 1,25-dihydroxyvitamin D3 or PMA express IE antigens, it is evident that not all cells that progress through the IE phase go on to produce infectious virus based on infectious center assays, indicating that there are additional blocks subsequent to IE expression that control the progression to the lytic phase in HCMV infected myeloid cells.

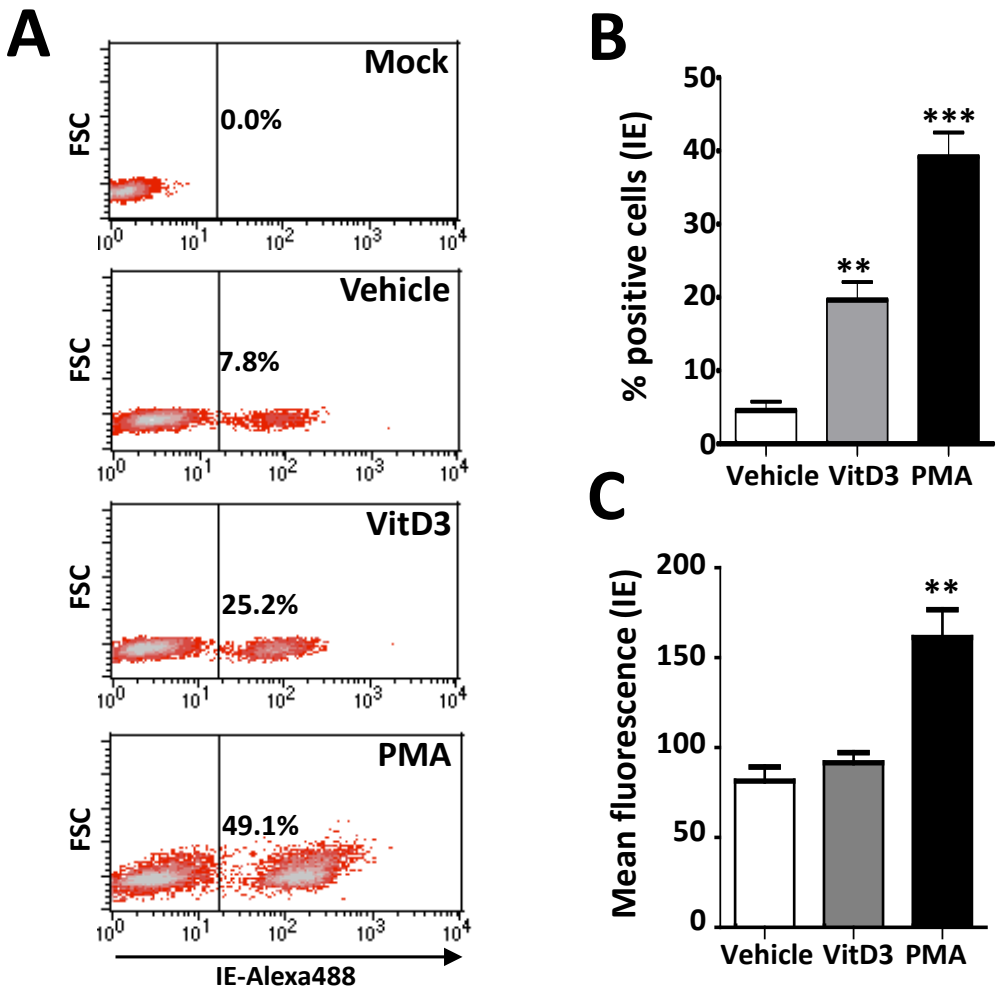



**Figure 5. 1,25-dihydroxyvitamin D3 treatment increases the percentage of cells supporting HCMV IE gene expression**

(A) THP-1 monocytes were treated with 1,25-dihydroxyvitamin D3 (100nM) or PMA (80nM) for 3 days and then infected with HCMV TB40E at a MOI of 10. At 1 day post-infection, cells were fixed, permeabilized and stained with anti-HCMV IE antibody mAB 810-Alexa488. Cells were analyzed by flow cytometry. (B) The percentage of IE positive cells at days 1 post-infection is presented graphically. The data are derived from four independent experiments including the one depicted in panel A (C) The mean fluorescence intensity of IE positive cells at days 1 post-infection is presented graphically. The data are derived from four independent experiments including the one depicted in panel A. VitD3, 1,25-dihydroxyvitamin D3. ** $p<0.01$,*** $p<0.001$.

### *HCMV early and late genes are expressed in 1,25-dihydroxyvitamin D3 stimulated cells*

Although IE expression is important for initiation of lytic infection, the expression of early and late genes are needed to complete the lytic phase (138,139). Therefore, the expression of early and late HCMV genes was examined by western blot in vehicle control, 1,25-dihydroxyvitamin D3 and PMA treated cells (Figures 6A and 6B). For these experiments, we analyzed UL44, a processivity factor associated with the viral DNA polymerase (140), which is expressed with delayed-early kinetics (141) and pp65, a tegument protein that is expressed with late kinetics (142). Vehicle control infected cells exhibited undetectable levels of either the delayed early UL44 or late pp65 proteins while cells treated with 1,25-dihydroxyvitamin D3 or PMA prior to infection showed dramatic upregulation of both UL44 and pp65.

Representative western blots are depicted in Figure 6A and the results are depicted graphically in Figure 6B in which the blots were quantitated and viral protein levels are shown relative to the cellular actin protein as a control. To further investigate viral gene expression patterns in these cells and determine what percentage of IE positive cells progress to the early phase as evidenced by UL44 expression, cells at days 1 and 4 post-infection were co-stained for IE1/2 and UL44 expression and analyzed by flow cytometry (Figure 6C). In vehicle treated cultures, about 7.5% of the cells were IE positive at day 1 post-infection, while the percentage of IE positive cells dropped to

4.4% at day 4 post-infection. Of the 4.4% IE positive cells at day 4 post-infection only 11% of cells were UL44 positive (0.5% of the total cellular population). In 1,25-dihydroxyvitamin D3 treated cultures, approximately 33% of the cells were IE positive at day 1 post-infection, while the percentage of IE positive cells declined to 19% at day 4 post-infection. However, of the 19% IE positive cells, 33% were also UL44 positive (6.5% of the total cellular population). In PMA treated cultures, 40% of the cells were IE positive at day 1 post-infection and the percentage decreased slightly to 38% at day 4 post-infection. Of the 38% IE positive cells, 48% were also UL44 positive (18% of the total cellular population). The results of these early and late gene expression profiling experiments are also in line with the results of infectious center assay and are all consistent with the conclusion that 1,25-dihydroxyvitamin D3 promotes HCMV lytic replication in myeloid cells.

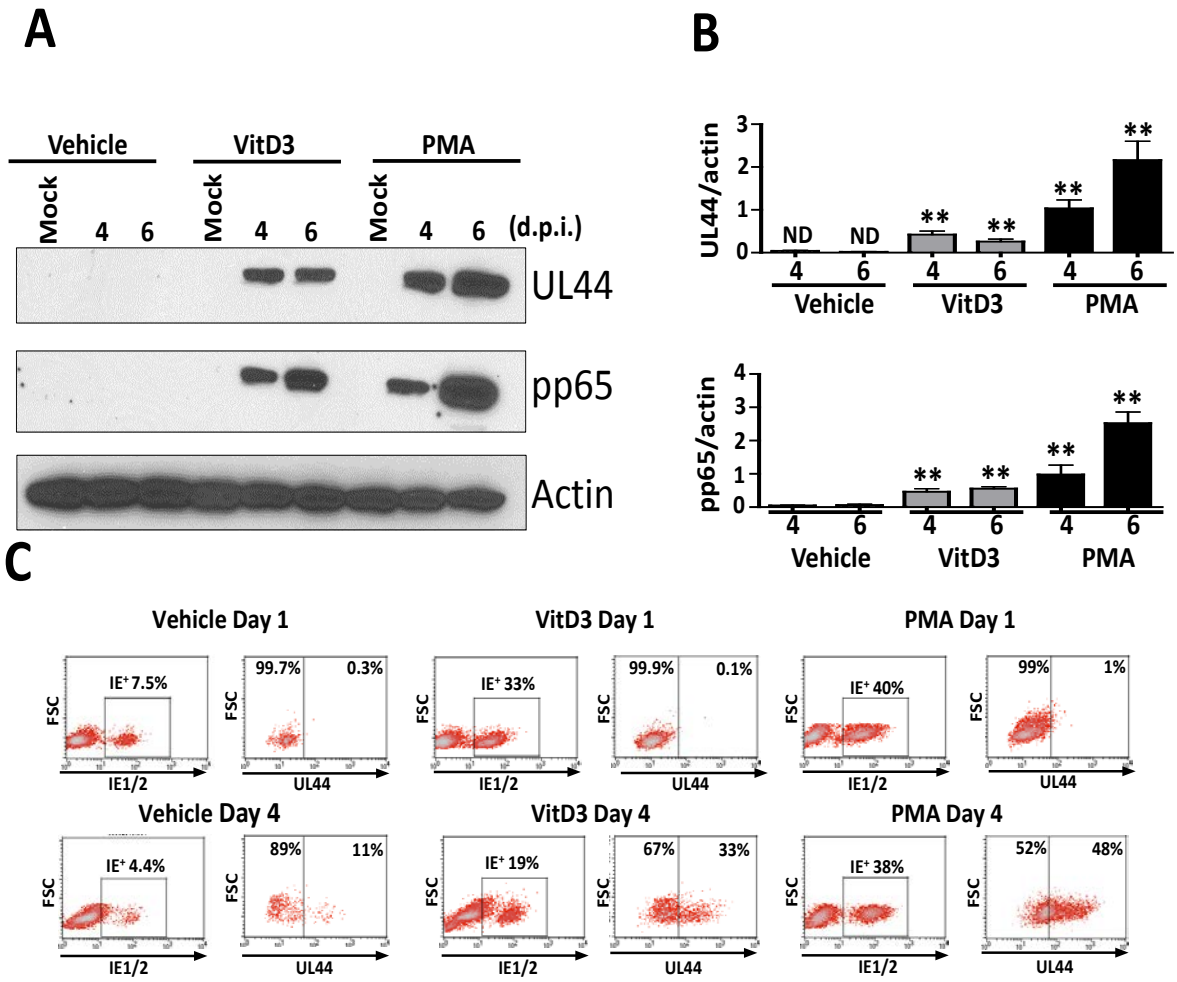



**Figure 6. 1,25-dihydroxyvitamin D3 promotes HCMV early and late gene expression**

(A) THP-1 monocytes were treated with 1,25-dihydroxyvitamin D3 (100nM) or PMA (80nM) for 3 days and then infected with HCMV TB40E at a MOI of 10. At the indicated times post-infection, cells extracts were analyzed by western blot for UL44 (early) and pp65 (late) gene expression. Western blot analyses demonstrated that UL44 and pp65 are robustly expressed in 1,25-dihydroxyvitamin D3 and PMA treated cells. Cell extracts were also analyzed for actin expression as an internal control. (B) The results of four independent experiments are shown graphically as the ratio of UL44 or pp65 to actin. (C) THP-1 cells treated as described in panel A were stained for IE and UL44 proteins at Days 1 and 4 post-infection and analyzed by flow cytometry. $IE^+$ cells were gated (left panel in each pair) and then plotted as a function of UL44 expression (right panel in each pair) to determine the fraction of $IE^+$ cells that have entered into the early phase as assessed by UL44 expression. The results shown are representative of 4 independent experiment. VitD3, 1,25-dihydroxyvitamin D3. , *p<0.05, **p<0.01.

***1,25-dihydroxyvitamin D3 uses a mechanism distinct from that of PMA to promote lytic replication***

Due to the fact that both 1,25-dihydroxyvitamin D3 and PMA can prime THP-1 cells to support lytic infection, it would be intriguing to determine if these two reagents deploy the same mechanism or if 1,25-dihydroxyvitamin D3 functions in a manner distinct from that of PMA. Moreover, since it is clear that IE protein expression is critical for the onset of lytic replication, we wished to investigate the effects of 1,25-dihydroxyvitamin D3 and PMA on IE gene expression in a more detailed manner. Based on published reports (124), it has been demonstrated that the HCMV IE enhancer region in THP-1 cells after infection is marked by histone 3 lysine 27 trimethylation (H3K27me3), and that the H3K27me3 mark at the IE enhancer is significantly decreased after PMA treatment. H3K27me3 is associated with a closed chromatin conformation and silenced gene expression (143), therefore it appears that decreased H3K27me3 in the IE region correlates with an open chromatin conformation and increased MIEP activity. We wanted to determine whether H3K27me3 associated with the IE enhancer is also decreased in 1,25-dihydroxyvitamin D3 treated cells. Using chromatin immunoprecipitation (CHIP) followed by PCR for the IE enhancer region, we find that the IE enhancer region in

control cells is in fact modified by H3K27me3 as reported by others (Figure 7A) (124,144). However, in both 1,25-dihydroxyvitamin D3 and PMA treated cells the CHIP-PCR signal is 4 to 10 fold weaker indicative of decreased H3K27me3 at the IE enhancer. Thus, these results are consistent with our analyses of IE1 protein expression and suggest that the transition of the MIEP enhancer region into an open conformation in 1,25-dihydroxyvitamin D3 treated cells is an important prerequisite for the transition to the lytic phase.

The MIEP contains binding sites for a number of transcription factors that are responsive to PMA such as NF-$\kappa$B and CREB (145), but it is unknown if the MIEP would be directly responsive to 1,25-dihydroxyvitamin D3. We cloned the HMCV IE promoter from the HCMV-FIX strain into the luciferase reporter pGL3 so that we could test whether 1,25-dihydroxyvitamin D3, like PMA would be able to directly stimulate the MIEP-luciferase reporter gene. We transfected THP-1 cells with pGL3-MIEP, stimulated cells with either 1,25-dihydroxyvitamin D3 or PMA, and measured luciferase activity. Reporter luciferase was internally controlled by comparison to constitutively expressed Renilla-luciferase. Interestingly, the Luciferase assay results demonstrate that while PMA can directly stimulate MIEP promoter activity, 1,25-dihydroxyvitamin D3 cannot (Figure 7B). These data are consistent with our flow cytometry data in Figure 5C, which indicated that 1,25-dihydroxyvitamin D3 treated

cells are more likely to support IE protein expression but do not exhibit increased levels of the IE protein on a per cell basis (Figure 5C). Thus, taken together, while both 1,25-dihydroxyvitamin D3 and PMA can promote an open conformation of the MIEP followed by IE protein production and onset of the lytic phase, the mechanisms used by the two inducers are not identical as the effects of PMA can at least be partially explained by a direct effect on the MIEP promoter.

Since the two inducers appeared to not utilize totally overlapping mechanisms we investigated whether the effects of 1,25-dihydroxyvitamin D3 would be additive regarding the onset of lytic phase. To test this hypothesis, THP-1 cells were treated with PMA or PMA + 1,25-dihydroxyvitamin D3 prior to infection. Culture media was harvested at multiple time points post-infection and viral titers were assessed by plaque assay. Compared to PMA treated cells, 1,25-dihydroxyvitamin D3 plus PMA did not show an additive effect as the kinetics and magnitude of the viral growth is the same (Figure 8). Thus, while the mechanisms are not totally overlapping, the convergence of the two compounds on creating an environment more suitable for IE protein expression seems to be a necessary prerequisite leading to the onset of the lytic viral life cycle.

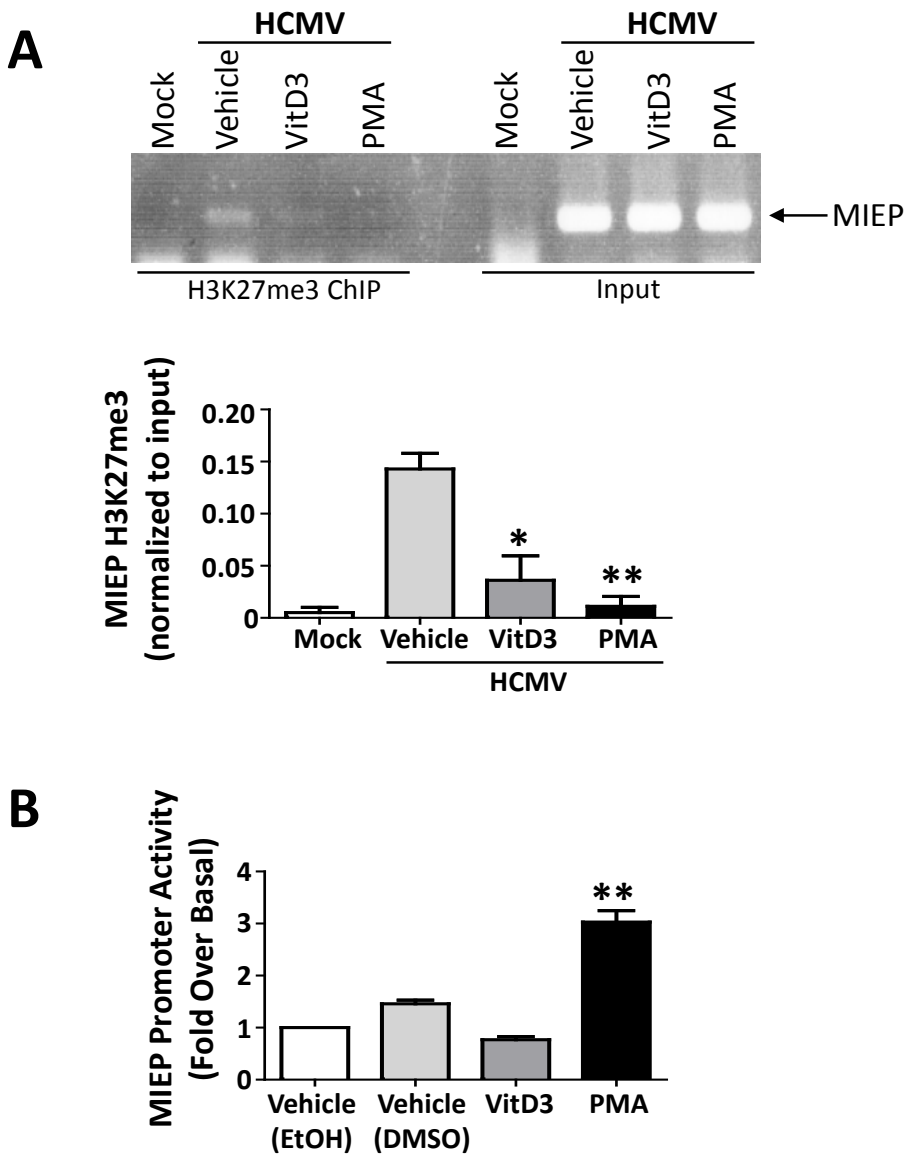



**Figure 7. 1,25-dihydroxyvitamin D3 promotes K27 demethylation of histone H3 associated with the HCMV IE enhancer region, but does not directly stimulate IE promoter activity**

(A) THP-1 monocytes were treated with 1,25-dihydroxyvitamin D3 (100nM) or PMA (80nM) for 3 days and then infected with HCMV TB40E at a MOI of 10. At 2 days post-infection, chromatin immunoprecipitation was used to examine the K27 methylation status of histone H3 associated with the HCMV IE enhancer region. The results presented are derived from 3 independent experiments. (B) MIEP-luciferase activity was assessed in transient reporter gene assays in the presence of 1,25-dihydroxyvitamin D3 or PMA. MIEP-luciferase activity was normalized to the internal control renilla luciferase. The results presented are derived from 3-5 independent experiments performed in duplicate. VitD3, 1,25-dihydroxyvitamin D3. *p<0.05, **p<0.01.

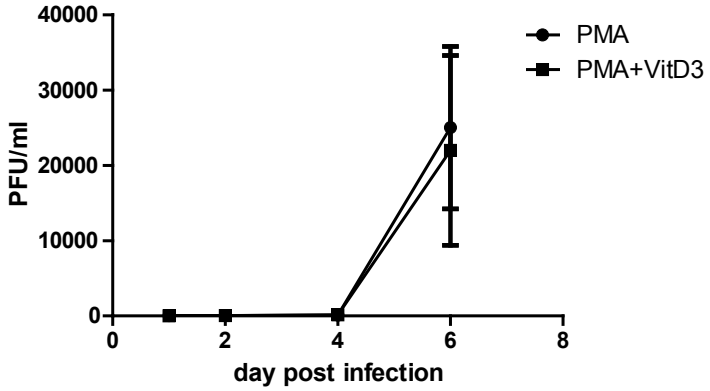



**Figure 8. 1,25-dihydroxyvitamin D3 and PMA do not function in a cooperative manner to increase lytic replication**

THP-1 cells were differentiated with PMA (80nM) or PMA (80nM) and 1,25-dihydroxyvitamin D3 (100nM) for one day, and then infected with HCMV at an MOI of 7. Cell supernatants were harvested at the indicated times post-infection and viral titers were determined by plaque assay on human foreskin fibroblasts. The results indicate that 1,25-dihydroxyvitamin D3 and PMA do not function cooperatively to enhance HCMV replication in THP-1 cells. The results are derived from 4 independent experiments. VitD3, 1,25-dihydroxyvitamin D3.

***The differentiation of THP-1 cells triggered by 1,25-dihydroxyvitamin D3 plays an important role in releasing the restriction on HCMV IE expression***

Based on published studies it is clear that 1,25-dihydroxyvitamin D3, like PMA, is an inducer of monocyte differentiation and maturation (34,99,118). However, while PMA induces the THP-1 cells to mature into a more highly differentiated macrophage-like state marked by strong adherence of the cells to plastic, 1,25-dihydroxyvitamin D3 induces differentiation into a mature monocyte marked by a non-adherent phenotype (118). Therefore, we wished to investigate the influence of monocyte differentiation properties on 1,25-dihydroxyvitamin D3 induced HCMV replication in the THP-1

model. Consistent with previous studies (118,119), we observed that PMA but not 1,25-dihydroxyvitamin D3 induced the appearance of a macrophage phenotype with flattened cells tightly adhered to the flask (Figure 9A). Immunostaining for the cell differentiation markers CD14, CD11b, CD36, CD54, and CD68 was also performed and the results demonstrate that 1,25-dihydroxyvitamin D3 consistently induces strong CD14 and moderate CD11b expression while having little to no effect on CD36, CD54, and CD68 expression (Figure 9B and Supplemental Figure 1). In contrast, PMA induced moderate levels of CD14, while inducing robust levels of CD11b, CD36, CD54, and CD68 (Figure 9B and Supplemental Figure 1). These results are entirely consistent with those of published studies and are further supportive of the conclusion that 1,25-dihydroxyvitamin D3 induces an intermediate differentiation phenotype typical of a mature monocyte, while PMA causes a terminally differentiated phenotype typical of a macrophage (118,146-148). Interestingly, while PMA induces terminal differentiation and halts cellular proliferation, 1,25-dihydroxyvitamin D3 treated cells continued to proliferate in agreement with previous studies (data not shown) (118).

We then asked whether there was a correlation between 1,25-dihydroxyvitamin D3 promoted differentiation and onset of lytic replication. Since 1,25-dihydroxyvitamin D3 appeared to not directly stimulate MIEP promoter activity, we hypothesized that the

effects of 1,25-dihydroxyvitamin D3 on IE expression might be delayed and only occur as the cells differentiated into mature monocytes. To test this postulate, THP-1 cells were treated with 1,25-dihydroxyvitamin D3 for different times (6 hours to 3 days) prior to infection with HCMV (experimental conditions are depicted graphically in Figure 10C). Expression of CD14 was monitored throughout the time course to assess cellular differentiation and IE positivity was assessed as a measure of the relative ability of the cells to promote lytic replication. IE expression was measured at 24 hours post-infection in all cases. This experimental system enables us to assess the expression of the differentiation marker CD14 and the HCMV IE protein in a series of cells in different stages of maturation. CD14 expression as measured by flow cytometry peaked at between 1 and 2 days post-stimulation with 1,25-dihydroxyvitamin D3 (Figure 10A). Interestingly, we did not observe significant effects of 1,25-dihydroxyvitamin D3 on IE positivity until 2 days post-stimulation with 1,25-dihydroxyvitamin D3 (Figure 10B). This finding is consistent with the conclusion that 1,25-dihydroxyvitamin D3-induction of lytic HCMV replication correlates with 1,25-dihydroxyvitamin D3 induced cellular differentiation. Moreover, the lack of a significant effect of 1,25-dihydroxyvitamin D3 at early time points argues against a direct effect of 1,25-dihydroxyvitamin D3 on the MIEP similar to what we observed in reporter assays in Figure 7.

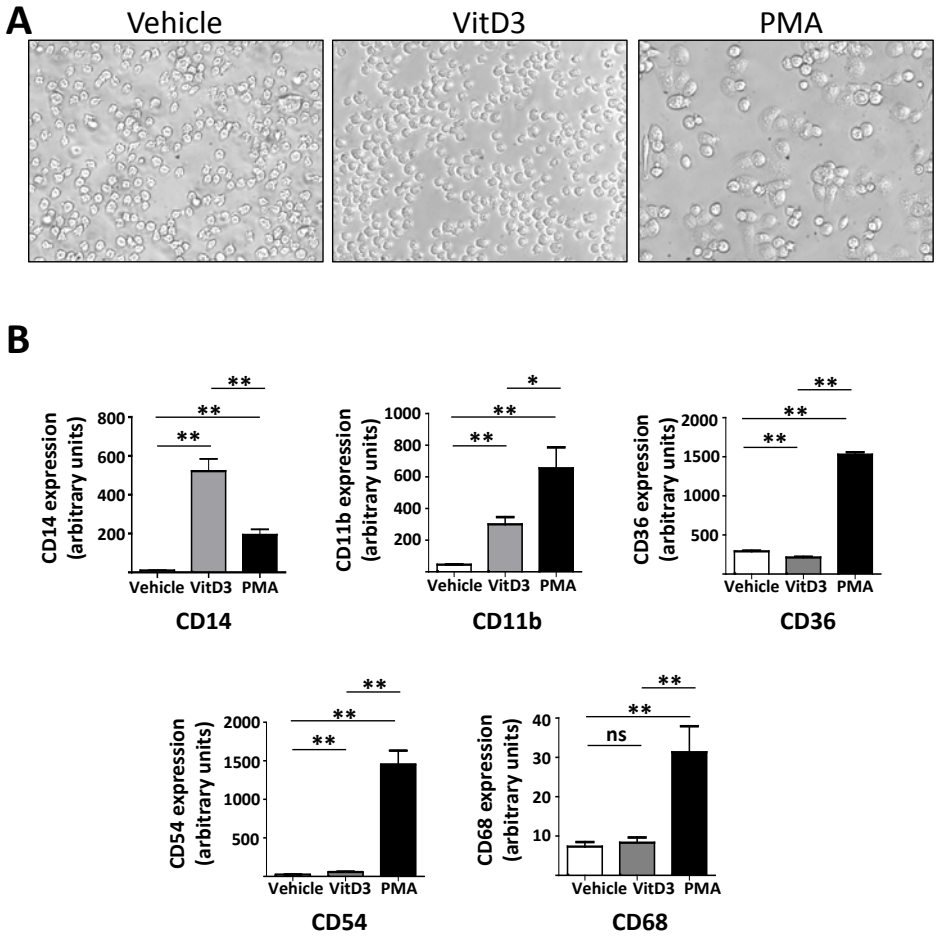
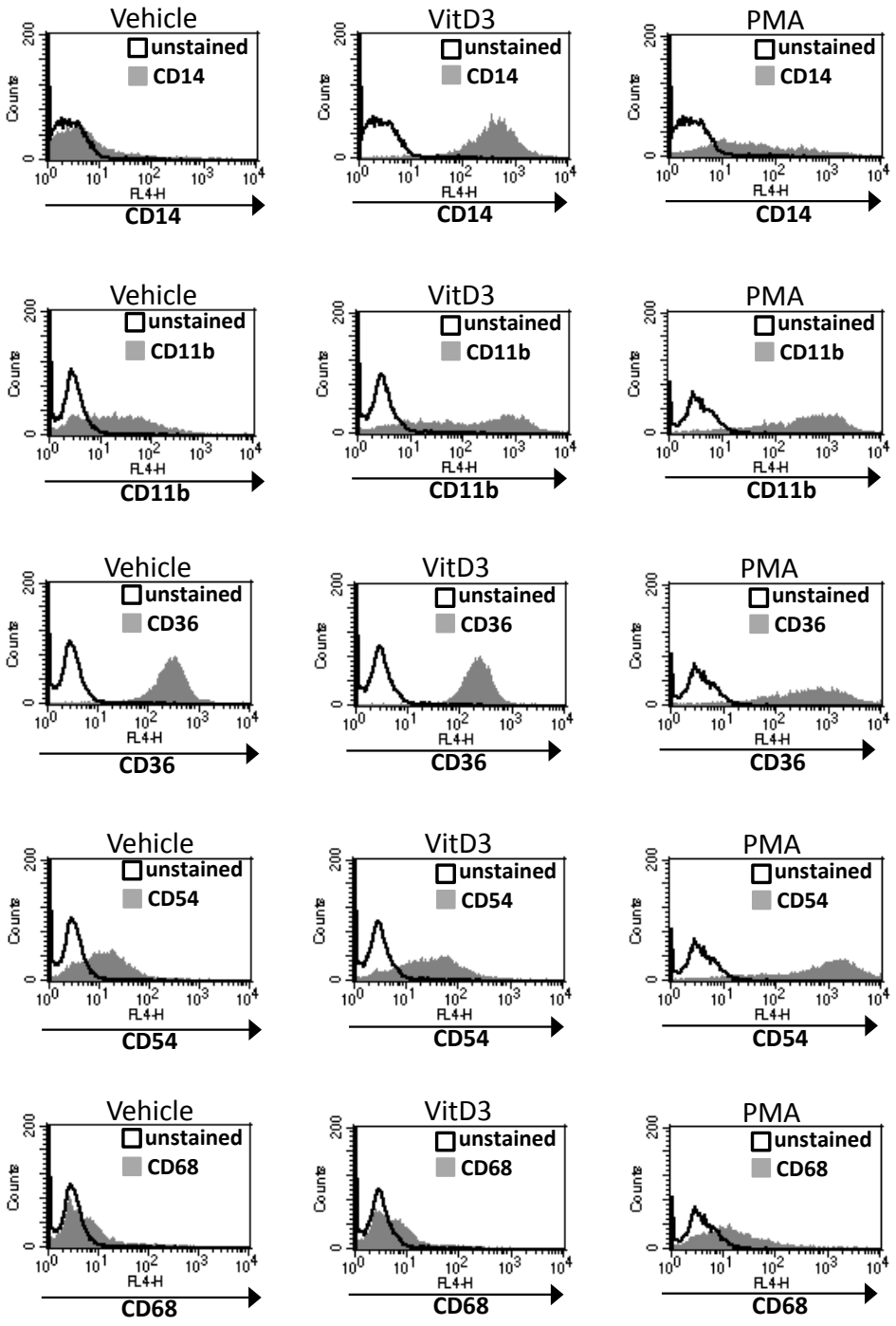



**Figure 9. 1,25-dihydroxyvitamin D3 and PMA induced THP-1 differentiation is phenotypically distinct**

(A) THP-1 cells were treated with 1,25-dihydroxyvitamin D3 (100nM) or PMA (80nM) for 3 days and photographed with a Olympus Q Color 5 camera equipped with QCapture Pro Software. (B) Cells treated as in panel A were stained with the inidcated antibodies and analyzed by flow cytometry. Data from four independent experiments is depicted graphically. VitD3, 1,25-dihydroxyvitamin D3. ** $p<0.01$.




**Supplemental figure 1.**

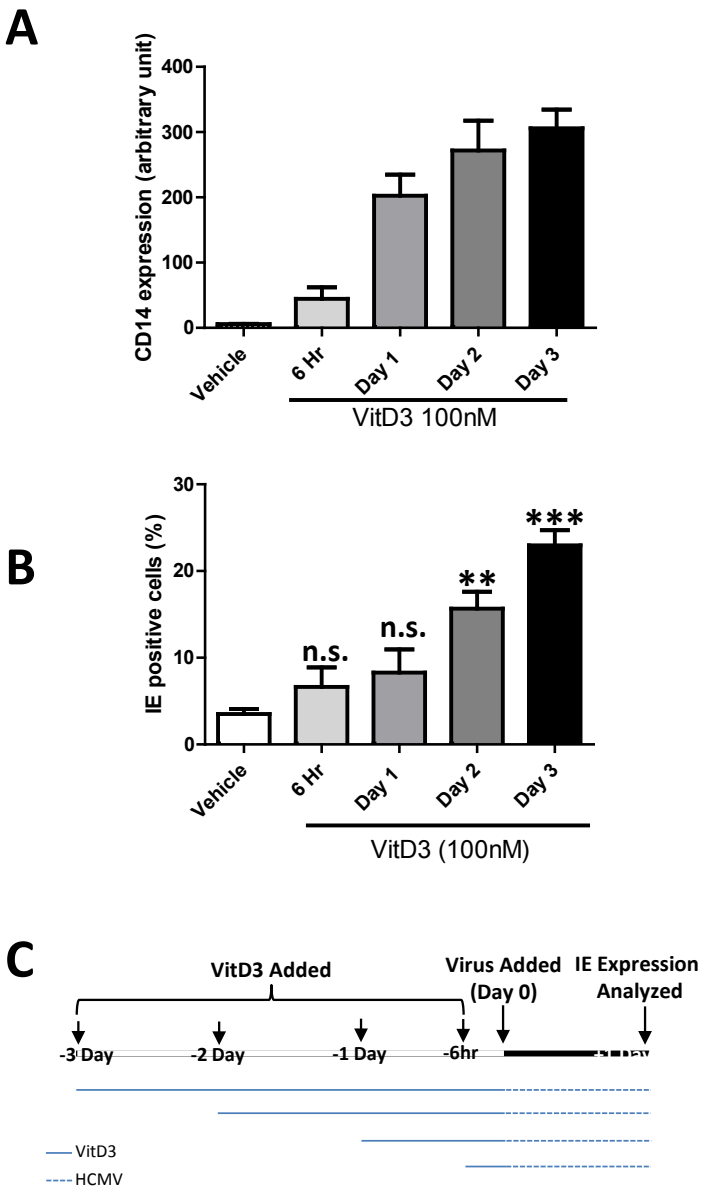



**Figure 10. The timing and magnitude of 1,25-dihydroxyvitamin D3 induced differentiation of THP-1 monocytes is important for supporting HCMV lytic infection**

(A) THP-1 cells were treated with 1,25-dihydroxyvitamin D3 (100 nM) for indicated times and CD14 expression was analyzed by flow cytometry. (B) Cells were treated with 1,25-dihydroxyvitamin D3 for the indicated times prior to infection with HCMV TB40E. At 24 hours post-infection cells were fixed, permeabilized and stained with HCMV IE antibody mAb810-Alexa488. The results represent flow cytometric analysis of IE positive cells and are derived from three independent experiments. (C) Experimental set up for the experiments depicted here. Cells were treated for variable lengths prior to infection with HCMV TB40E. In all cases IE gene expression was analyzed at 24 hours post-infection. VitD3, 1,25-dihydroxyvitamin D3. **p < 0.01, ***p < 0.001.

## Discussion

In this study, we found that 1,25-dihydroxyvitamin D3, a hormone present in the circulatory system and in many tissues (149), can promote HCMV replication in primary peripheral blood monocytes and in THP-1 cells. Our data are consistent with a mechanism whereby 1,25-dihydroxyvitamin D3 induced HCMV replication involves the induction of monocyte differentiation. Based on our data, we propose that monocyte maturation/differentiation induced by 1,25-dihydroxyvitamin D3 leads to a modification of histone 3 K27 methylation in the HCMV IE enhancer region, which results in a conversion to an open chromatin conformation and induction of IE gene expression. Moreover, since 1,25-dihydroxyvitamin D3 does not promote cell cycle arrest and terminal differentiation of monocyte cell lines in vitro (118), this system represents an interesting paradigm that could be utilized to study reactivation of virus in model systems. In particular, while previous studies have shown that PMA differentiated THP-1 macrophages can support permissive infection, in this study we found that monocytes in the transition between mature monocytes and macrophage stages, like the state induced by 1,25-dihydroxyvitamin D3, can also support permissive infection.

Our work is the first to explore the effect of 1,25-dihydroxyvitamin D3 on HCMV in myeloid cells. Although the concentration of 1,25-dihydroxyvitamin D3 used

in our study is somewhat higher than the concentrations found *in vivo* (150), published studies have demonstrated that in hematopoietic progenitor cells derived from umbilical cord blood, a similar dose of 1,25-dihydroxyvitamin D3 added once a week has the same effects on monocyte-macrophage differentiation as does physiological concentrations of 1,25-dihydroxyvitamin D3 supplemented daily (150). In this case the difference between the two conditions is that high dose treatment causes more rapid differentiation and lineage commitment than does physiological concentrations and thus the high doses like those used in our study simply facilitate experiments performed in an *in vitro* setting.

Although in this research, our main focus is to define the effect of 1,25-dihydroxyvitamin D3 on HCMV lytic replication in peripheral blood monocytes, an equally attractive question to be addressed in the future is to ask what effect 1,25-dihydroxyvitamin D3 has on HCMV replication in hematopoietic progenitor cells (HPCs), a cell type well-known as a reservoir for HCMV latency (72,151). In particular does 1,25-dihydroxyvitamin D3 treatment of HPCs affect the ability of HCMV to choose a latent or lytic path and would the presence of high concentrations of 1,25-dihydroxyvitamin D3 promote lytic reactivation? It is known in the HCMV field that macrophages and mature dendritic cells can support lytic infection but hematopoietic progenitor cells, myeloid progenitor cells and monocytes are cell types

known to typically establish a latent infection (19,87). THP-1 cells treated with 1,25-dihydroxyvitamin D3 do not show a phenotype characteristic of mature macrophages and therefore, 1,25-dihydroxyvitamin D3 appears to induce a differentiation state in between monocytes and macrophages. Previously, studies of HCMV in myeloid cells have focused on hematopoietic progenitor cells (23,152), myeloid progenitor cells (27,121), monocytes (22,80,91) or macrophages (153), and thus our research offers some additional insight into HCMV replication in cells that are transitioning between the monocyte and macrophage stages. Although previous studies suggest that HCMV typically enters the lytic phase in mature macrophages (42,92,136) our results indicated that cells transitioning between the monocyte and macrophage stages can also support lytic infection. HCMV infection itself has been shown to promote monocyte differentiation but the differentiation patterns triggered by infection alone are not typically capable of efficiently driving lytic infection (80,91). It is highly possible that there is a differentiation threshold that is needed to be passed in order to appropriately kindle a lytic infection. 1,25-dihydroxyvitamin D3 treatment may prime the cells in the differentiation process, and when infection provides the appropriate additional differentiation signals, the threshold is surpassed.

In these studies we examined whether 1,25-dihydroxyvitamin D3 differentiated monocytes can support lytic infection, but as mentioned above an important question

to ask in the future is whether 1,25-dihydroxyvitamin D3 may also be involved in the reactivation of HCMV from latency. Recently, glucocorticoids have been shown to trigger reactivation of HCMV in primary monocytes through direct activation of the IE promoter (105). Since there are studies showing crosstalk between glucocorticoids and 1,25-dihydroxyvitamin D3 (154), and since 1,25-dihydroxyvitamin D3 can enhance glucocorticoid action in human monocytes (155,156), it is reasonable to speculate that 1,25-dihydroxyvitamin D3 may also be involved in the regulation of HCMV reactivation or work in concert with glucocorticoids in this process. Perhaps since the mechanisms of actions appear to be disparate (glucocorticoids directly on the MIEP and 1,25-dihydroxyvitamin D3 on cellular differentiation) these two hormones may work synergistically to affect HCMV replication and/or reactivation.

Differentiation of monocytes by 1,25-dihydroxyvitamin D3 appears to robustly induce the HCMV lytic phase, but the precise molecular mechanism(s) that regulates this activity remain unknown. Based on our studies, we postulate that signaling activity and gene expression patterns typically triggered by 1,25-dihydroxyvitamin D3 to drive monocyte maturation/differentiation are required for HCMV lytic infection to proceed. Global comparison of downstream gene expression induced by 1,25-dihydroxyvitamin D3, PMA and/or glucocorticoids in monocytes by microarray or

RNA-seq will be helpful to narrow down the list of candidates, and increase the likelihood of identifying the essential molecules that regulate this switch.

From a clinical perspective, there are many ongoing studies showing that 1,25-dihydroxyvitamin D3 or its synthetic analogs can have anti-cancer effects, and can have beneficial effects on cardiovascular or autoimmune disease (157-159). Since there is some evidence supporting a role for HCMV infection in the progression of cancers (160,161), cardiovascular and autoimmune diseases (84,162), our study could prompt questions regarding whether or not 1,25-dihydroxyvitamin D3 and its synthetic analogs should be used therapeutically. In addition, the effect of 1,25-dihydroxyvitamin D3 on HCMV replication in other cell types including cancer cells is still unknown. Moreover, while 1,25-dihydroxyvitamin D3 can have genomic and non-genomic effects (163), whether those synthetic analogs have the same effect of 1,25-dihydroxyvitamin D3 on HCMV replication is another intriguing question to answer and which may provide an important tool to tease out the specific pathways involved in lytic induction.

**Acknowledgements**

We would like to thank Christine O'Connor for providing the recombinant TB40E virus expressing mCherry and J. Shanley for UL44 antibody. We thank the Cell Processing and Manipulation Core in the Translational Cores, and Physicians and Nurses at CCHMC for obtaining and processing peripheral blood samples for monocyte purification. We also thank the CCHMC Translational Research Trials Office for providing the regulatory and administrative support for this endeavor. S.E. Wu was supported by a Teaching Assistantship at the University of Cincinnati. This work was supported by National Institutes of Health Grants R01-AI058159 and R56-AI095442 awarded to W.E.M.

# Chapter II Appendix:

# Unpublished studies pertaining to Vitamin D and Cytomegalovirus Replication

## Material and methods

### Vitamin D receptor knockout mice

VDR knockout mice on a Balb/c background were obtained from Glendon Zinsser at the University of Cincinnati. Mice were maintained in the vivarium on 12 hour light and 12 hour dark period. Mice were fed with special diet chow (20% lactose, 2% calcium and 1.25% phosphorus) to maintain phosphorus and calcium homeostasis and allowed access to chow and water *ad libitum*. Heterozygous males and females were used for breeding and genotypes were screened by PCR using primers and protocols published on the Jackson Laboratory website.

http://jaxmice.jax.org/protocolsdb/f?p=116:2:0::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:1160,006133

### MCMV infection of VDR knockout mice

3-4 month old female mice were infected via intraperitoneal injection of $10^6$ pfu MCMV pARK25 virus per animal. At day 4 post-infection, infected mice were sacrificed. Spleens were isolated, homogenized and used for plaque assay.

**MCMV plaque assays**

$10^5$ NIH 3T3 cells were plated into each well of 12 well plates and maintained in DMEM supplemented with 10% calf serum, and penicillin and streptomycin as described previously. 1 μl or 10 μl tissue homogenate was added to each well to infect NIH 3T3 for 3 hours in 37℃ 5% CO2. After infection, inocula were removed, and CMC/MEM (1:1) mixture was overlaid on NIH3T3 monolayer. Cells were incubated in cell culture incubator for five days to allow plaques to develop. On day 5, cells were fixed with methanol, and stained with Giemsa. Plaque numbers were quantified using a dissecting microscope.

**General reagents**

Human TGF-β was generously provided by Dr. Thomas Thompson. Anti-HCMV IE antibody conjugated to Alexa 88 was purchased from Millipore. Anti-Human CD14 antibody conjugated to APC was obtained from eBioscience.

**TGF-β treatment**

THP-1 cells grown as described above were treated with 10 ng/ml TGF-β, 10 ng/ml TGF-β combined with various concentrations of 1,25-dihydroxyvitamin D3, or vehicle for designated time periods prior to infection.

## Results

### Replication of murine cytomegalovirus (MCMV) in VDR knockout BALB/c mice

Since our results demonstrated that 1,25-dihydroxyvitamin D3 can promote HCMV replication in monocytes in vitro, we wished to determine whether 1,25-dihydroxyvitamin D3 would also affect murine cytomegalovirus (MCMV) replication and hypothesized that replication of MCMV might be increased in animal models of MCMV infection. We used vitamin D receptor (VDR) knockout mice on the Balb/c background to examine this question. Mice were intraperitoneal injected with MCMV strain pARK25 at a MOI of $1x10^6$ pfu per animal. At day 4 post-infection, VDR knockout mice (-/-), heterozygous (+/-) and wildtype (WT) mice were sacrificed, and

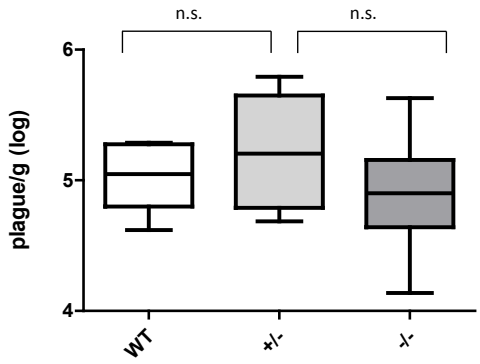



**Figure 11. Murine cytomegalovirus replicates to a similar degree in spleens of wildtype and vitamin D receptor knockout mice.** Mice were infected i.p. with the MCMV pARK25 strain at $10^6$ pfu per animal. At day 4 post-infection, mice were sacrificed, and spleens were harvested. Spleen homogenates were used to infect NIH 3T3 cells for plaque assay. WT: wildtype, +/-: heterozygous mouse, -/-: knockout mouse, n.s.: not statistically significant.

spleens were isolated, and homogenized. Homogenates were used for plaque assay to determine viral titers (Figure 11).

The results indicated that infected VDR knockout mice did not display significant differences in viral titers in the spleen suggesting that the VDR is not critical for MCMV replication *in vivo*. However, while innate immune response should happen very fast after infection (164,165), whether there is a difference in an earlier time-point after infection is unknown. In addition, although it is known that vitamin D can regulate immunoresponsiveness in both human and mouse(166,167), there are some differences in the genes that are regulated by vitamin D when comparing mouse and human. For example, in humans cathelicidin (LL-37) is a gene that is well known to be regulated by vitamin D with a demonstrable VDR binding site in the promoter region, but in mouse, there is no VDR binding site in the promoter area (168). Therefore, it is also possible that vitamin D may not have an effect in MCMV replication in murine myeloid cells, while having a profound effect on HCMV replication in humans. Further studies will be needed to clarify these issues.

**The influence of TGF-$\beta$ on HCMV replication in 1,25-dihydroxyvitamin D differentiated THP-1 cells**

It is known that TGF-β can act synergistically with vitamin D to promote

monocyte-macrophage differentiation (47-49). Therefore, we hypothesized that TGF-β and vitamin D may function together to synergistically enhance HCMV replication in monocytes. We first repeated the experiments regarding the effects of TGF-β and 1,25 -dihydroxyvitamin D3 on monocyte differentiation (Figure 12, panel A). When THP-1 cells were treated with 1 nM of 1,25 -dihydroxyvitamin D3 alone for 3

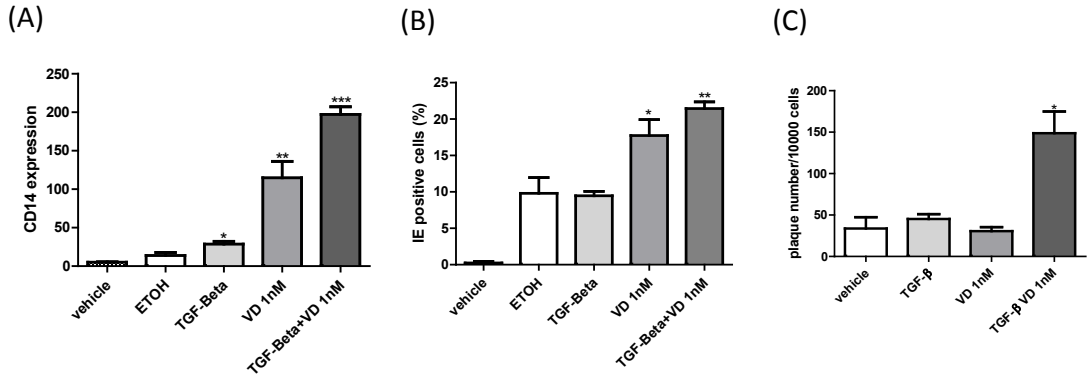



**Figure 12. TGF-β combined with 1,25-dihydroxy vitamin D3 promotes THP-1 differentiation and HCMV replication**

(A) THP-1 cells were treated with vehicle (EtOH), TGF-β (10 ng/ml), vitamin D, or TGF-β plus vitamin D for 3 days, and CD14 was analyzed by flow cytometry. (B) THP-1 cells were treated as mentioned in (A), and infected with TB-40 E virus at MOI of 10. At day 1 post-infection, cells were harvested, and stained with anti-HCMV IE antibody conjugated to Alexa-488. The percentage of IE positive cells was assessed by flow cytometry. (C) At day 6 post-infection, 10,000 THP-1 cells/sample were harvested and co-cultured with human foreskin fibroblasts for 2 days. After 2 days of co-culture, fibroblast monolayers were overlaid with CMC/MEM and incubated for 8 days to allow for plaque development. The data represent 3 independent experiments. * :p<0.05, ** :p<0.01, *** : p<0.001

days, CD14 expression is induced by 20 fold, while10 ng/ml TGF-β treatment alone induced CD14 expression by 5 fold. TGF-β plus vitamin D caused the strongest CD14 expression, and when comparing with control THP-1 cells, the CD14 expression was

increased by almost 38 fold (Figure 12A). These results are consistent with the previous observations that TGF-β and vitamin D together can promote increased monocyte-macrophage differentiation (47). We then examined whether the combination of TGF-β and vitamin D would enhance HCMV gene expression and/or lytic replication. TGF-β treatment alone did not increase the percentage of THP-1 cell expressing HCMV IE, but vitamin D alone or vitamin D plus TGF-β increased the percentage of THP-1 cells expressing HCMV IE (Figure 12B). TGF-β alone or 1 nM vitamin D alone did not lead to increases in viral replication in infectious center assays but TGF-β plus vitamin D causes a 4 fold increase in viral replication (Figure 12 C). It should be noted that we used a more dilute concentration of Vitamin D (1nM) than in earlier studies in this chapter (100nM) to assess the influence of TGF- β on Vitamin D induced monocyte differentiation and HCMV replication. The results from this experiment further supported that the conclusion that the differentiation state of THP-1 cells is crucial for triggering HCMV permissive infection. Whether TGF-β plus vitamin D can also promote HCMV replication in primary monocytes is still unknown, and it will be an interesting future direction for the research.

# Chapter III

# Pharmacological dissection of US28, M33 and ORF74 vGPCR signaling pathways using the YM-254890 G$\alpha$q inhibitor

Shu-en Wu, Fabiola M. Bittencourt and William E. Miller

***Unpublished Observations***

**Abstract**

Herpesviruses are ubiquitous in the human population and can establish life-long latency in their hosts. The β- and γ-herpesviruses encode viral G protein-coupled receptors (vGPCRs) in the viral genomes. vGPCRs are structurally similar to chemokine receptors and can modulate and evade host responses presumably to facilitate viral survival and dissemination. Human cytomegalovirus encoded US28, mouse cytomegalovirus encoded M33, and Kaposi's sarcoma-associated herpesvirus encoded ORF74 vGPCRs all can constitutively trigger Gαq dependent signal transduction. Moreover, it is known that US28, M33, and ORF74 can all activate NF-κB, CREB, and NFAT, but whether the activation of these downstream signaling molecules is Gαq dependent or not is unclear. Because of the potential role that these vGPCRs play in viral pathology and chronic diseases and the possibility that these vGPCRs could represent novel drug targets, it is important to dissect the signaling pathways initiated by vGPCRs using available pharmacological inhibitors. In this research, YM-254890, a pharmacological Gαq/11 specific inhibitor was used to determine which downstream signaling molecules are Gαq dependent. Using the YM-254890 compound, we demonstrate that the HCMV encoded US28 and MCMV encoded M33 vGPCRs use a similar mechanism to activate phospholipase C and downstream signaling molecules including NFAT, NF-KB, and CREB while the KSHV encoded ORF74 uses a distinct signal mechanism to activate the same set of signal

molecules. Moreover, the YM-254890 effectively blocks US28 triggered signaling in HCMV infected cells demonstrating the utility of this compound. Finally, based on these results, we conclude YM-254890 is a potent and specific tool that can be more fully exploited for exploring the signaling and function of herpesvirus encoded GPCRs both *in vivo* and *in vitro*.

## Introduction

β- and γ- herpesviruses encode G protein-coupled receptors (vGPCR) homologs in their genomes (50,51). These vGPCRs contain all of the components of a classical GPCR including 7 transmembrane domains, an extracellular amino terminus, and an intracellular carboxy terminus and are most structurally similar to mammalian chemokine receptor subfamily of GPCRs (51). Cellular chemokine receptors control migration of leukocytes to sites of inflammation, control migration of hematopoietic cells out of the bone-marrow lymphoid (169,170)and control movement of a number of additional cell types (171,172). Published studies have shown that unlike cellular chemokine receptors, which respond to specific chemokines, and are typically coupled to Gαi/o (173,174), vGPCRs bind promiscuously to multiple chemokines, exhibit high levels of constitutive activity and may even be coupled to more than one type of G-protein (68,175,176).

The members of the β- and γ- herpesviruses establish latency following primary infection and utilize multiple mechanisms to modulate cellular regulatory pathways thus enabling the viruses to maintain this latent state within the host(177-179). The herpesvirus vGPCRs are thought to activate cellular signal transduction pathways to facilitate the dissemination of the viruses, facilitate viral growth within specific tissue sites, and/or control the lytic/latent switch (50,180).

US28 is one of four vGPCRs encoded by the β-herpesvirus human cytomegalovirus (HCMV) (56). US28 can bind to multiple chemokines including MCP-1, RANTES, MIP-1α, and Fractalkine (56). Previous studies have demonstrated that US28 can trigger ligand dependent and ligand independent signaling pathways (68,181), and it can also function as a chemokine sink to reduce the concentration of chemokines surrounding the infected cells (59). However, research has shown that US28 deficient HCMV mutants do not show defects in viral replication in human foreskin fibroblasts in vitro (182) and more studies will be required to begin to ascertain the functional role that this protein plays in viral pathogenesis in vivo and identify the cell types in which US28 plays an important role during infection. US28 has been implicated as an active participant in HCMV associated pathologies such as atherosclerosis and glioblastoma (183,184). In this regard, US28 has been shown to promote the migration of vascular smooth muscle cells and macrophages and as such may play an important role in the initiation of atherosclerosis(57,58). Moreover, US28 can activate cellular proliferation pathways and induce cellular transformation (185-188) suggesting that US28 may facilitate the progression of HCMV associated tumors such as glioblastoma (189). HCMV establishes latency in hematopoietic stem cells, myeloid progenitor cells, and monocytes (190) and it is interesting that expression of US28 can be detected in these cells in both *in vivo* and *in vitro* infection

models (Chapter IV). The function of US28 in latency remains unknown, but it is interesting to speculate that US28 may play a role in the establishment or maintenance phases of latency (179). On the other hand, there are studies showing that transient expression of US28 can stimulate the promoter of the HCMV immediate-early (IE) gene (191,192) suggesting that US28 could participate in HCMV reactivation since the expression of IE gene is a critical step for HCMV reactivation (193). Based on all those reasons, US28 is an attractive target for therapeutic intervention as blocking US28 signaling activity could intervene in aspects of both pathogenesis and HCMV related pathology.

The highly related mouse cytomegalovirus (MCMV) encodes two vGPCRs termed M33 and M78(194). M33, like US28, exhibits high levels of constitutive signaling activity(195); however ligands for this vGPCR have yet to be identified. Published studies by the several laboratories including ours have indicated that M33 deficient MCMV replicates with wildtype efficiency *in vitro* but exhibits a severe replication defect in the salivary gland *in vivo (60,196)*. The salivary gland is an important site of persistent virus replication and can shed infectious virus into the saliva for weeks to months following a primary cytomegalovirus infection(197). The salivary gland phenotype exhibited by the ΔM33 virus experiments suggest that cytomegalovirus vGPCR signaling may have an important role in host-host

transmission occurring via the salivary route. Whether US28 plays a similar role in HCMV persistent infection in human salivary glands is still elusive, although research has shown that insertion of US28 into M33 deficient MCMV genome can partially rescue the phenotype in mouse salivary glands(198).

The Kaposi's sarcoma-associated herpesvirus (KSHV) encodes one vGPCR in its genome termed ORF74 (52). ORF74 binds to multiple chemokines in the IL-8 family including IL-8 itself and GRO-$\alpha$ (199). ORF74, like US28, can trigger both ligand-dependent and ligand-independent signaling pathways(50). ORF74 is particularly noted for its ability to drive increased transcription and secretion of vascular endothelial growth factor (VEGF) thus promoting angiogenesis(54). Kaposi's sarcoma tumors are highly vascularized (54,55) and thus the angiogeniesis promoting effects of ORF74 have classified it as an oncogene in this setting. From a pathogenesis perspective, the ORF74 homolog in MHV-68 has been demonstrated to affect viral reactivation from latency (53). Therefore, ORF74 appears to be a central player in multiple aspects of KSHV pathogenesis and disease.

Perhaps one common thread that seems to arise when comparing the biological activities of the herpesvirus vGPCRs is their abilities to regulate latency and persistence and therefore the identification of pharmacological agents targeting common aspects of the signaling activities of these molecules may provide novel

therapeutics to regulate herpesvirus latency and persistence.

Based on previous studies, US28, M33, and ORF74 are known to be constitutively (e.g. without agonist) coupled to Gαq and capable of activating a similar repertoire of downstream signaling molecules including PLC-β, CREB, NF-κB, and NFAT(63,66,67,195). In the case of M33, mutational analysis of the NRY G-protein coupling motif has indicated that the activation of NF-κB occurs via a G-protein independent mechanism(63), but in the case of US28 and ORF74 whether all the downstream signaling molecules are Gαq dependent is unknown. Moreover, studies designed to address this topic to this point have not utilized Gq inhibitors to explore this question. In some previous studies, the overexpression of individual heterotrimeric G proteins has been used to determine which G protein complex is engaged by the vGPCRs(68,200). However, the possibility that such an approach may initiate the cross-talk or aberrant coupling is a distinct possibility. In the current study, we explored using a Gαq specific inhibitor to examine the G-protein dependency involved in a broad range to signaling pathways kindled by these vGPCRs.

YM-254890 is a Gαq/11 specific pharmacological inhibitor isolated from Chromobacterium sp. QS3666 (25). YM-254890 was initially identified as an inhibitor of ADP-induced platelet aggregation (201). Subsequently, it was shown that this

compound inhibits ADP-induced platelet aggregation via its ability to specifically inhibit Gαq/11. YM-254890 is a cyclic depsipeptide that can bind to GDP bound form of Gαq and lock it into its inactive form. In light of the activation of multiple signaling molecules by the vGPCRs, and the involvement of vGPCRs in the pathology of many chronic diseases, it is crucial to not only identify the pathways utilized by the vGPCRs to activate downstream signaling pathways, but also to evaluate compounds that could be of potential therapeutic value.

In this research, YM-254890 was used to determine which downstream signaling activities of US28, M33 and ORF74 are Gαq/11-depedent. As expected, based on studies of US28 coupling in Gαq deficient MEFs, YM-254890 inhibits US28 and M33-induced accumulation of 1,4,5 inositol-trisphosphate ($IP_3$) in a dose dependent manner, confirming that these cytomegalovirus vGPCRs are coupled to PLC-β via a Gq dependent mechanism. Surprisingly YM-254890 does not have an inhibitory effect on ORF74-induced $IP_3$ accumulation suggesting that promiscuous coupling of ORF74 to Gαi (200) may be responsible for this signaling. For downstream signaling molecules, the inhibition of the activation of CREB by US28 and M33 is YM-254890 dose dependent and coincides with the concentrations necessary to block Gαq activity. In contrast both US28 and M33 signaling to NF-κB is resistant to YM-254890 indicating that the activation of NF-κB is Gαq/11 independent.

This result confirms previous studies regarding M33 and NF-κB activation and suggests that both US28 and M33 use a novel G-protein independent mechanism to stimulate NF-κB signaling activity. US28-and M33-induced activation of NFAT is only modestly affected by YM-254890 indicating that NFAT like NF-κB likely uses a mechanism distinct from Gαq.

Taken together, these studies highlight the complexity of herpesviral vGPCR signaling and demonstrate that while the receptors can clearly be coupled to Gαq to activate downstream signaling, there exists multiple pathways emanating from these receptors that are likely to mediate all of the biological effects of the vGPCRs. However, these studies do demonstrate that several important activities of the cytomegalovirus GPCRs are clearly inhibited by the YM-254890 compound and that this compound will be useful in functional assays to define critical aspects of vGPCR mediated viral pathogenesis. In particular, the ability of YM-254890 to inhibit multiple aspects of MCMV-M33 signaling is particularly interesting and will be useful to begin to correlate mechanisms of signaling with in vivo biological activities such as viral persistence in the salivary gland.

## Materials and methods

**Cell culture.** HEK cells were maintained in DMEM with 4.5 g/L glucose and L-glutamine without sodium pyruvate supplemented with 10% fetal bovine serum, and penicillin and streptomycin at 37℃ 5% carbon dioxide. Human foreskin fibroblasts were cultured in DMEM with 4.5 g/L glucose and L-glutamine without sodium pyruvate supplemented with 10% FetalClone III, and penicillin and streptomycin at 37℃ 5% carbon dioxide.

**Transient transfection.** For transient expression of US28, M33 and ORF74 in HEK cells, 15 ng of pcDNA3 plasmid containing the indicated vGPCR was co-transfected with 250 ng of pcDNA3 vector into HEK cells.

**Luciferase assays.** 200,000 cells were plated into each well in 12-well plates, and cultured overnight before transfection. For stable HEK cell lines, 250 ng pcDNA3, 10 ng of pFA2CREB, 30 ng pFRLUC, and 15 ng of phRGTK-renilla were used in transfection for the assessment of CREB driven luciferase activity. 250 ng pcDNA3, 15 ng pMHCLUC, and 15 ng phRGTK-renilla were transfected into stable cell lines for determining NF-κB induced reporter activity. For NFAT report assays, 250 ng pcDNA3, 15 ng pGL3 9xNFATLUC, and 15 ng phRGTK-renilla were transfected.

YM-254890 or DMSO (solvent control) was added at the time of transfection. YM-254890 was a gift from Astellas Pharma Inc (Ibaraki, Japan). 48 hours after transfection, cells were washed with 1X DPBS, and lysed in 200 µl 1X passive cell lysis buffer. After 15 minutes incubation at room temperature, 10 µl of cell lysate was used for reporter assays. 50 µl luciferase substrate was added to cell lysate and luciferase activity was measured by Glomax 20/20 luminometer (Promega). For renilla measurement (internal control), 50 µl Stop & Glow reagent was added to the cell lysate, and the intensity was determined by Glomax 20/20 luminometer (Promega). To assess reporter gene activity, experimental firefly-luciferase readings from control cells were divided by the control renilla-luciferase readings from the same cells and defined as 1. Fold induction is calculated by dividing the firefly-luciferase/renilla-luciferase ratio of cells expressing the various vGPCRs in the presence or absence of indicated concentration of YM-254890 by the firefly-luciferase/ renilla-luciferase ratio in control cells.

**$IP_3$ accumulation assays.** 12-well cell culture plates were coated with collagen and 200,000 HEK cells were plated into each well. 1 µCi of $^3$H-myoinositol was added to each well to label the cells at 37°C and 5% $CO_2$ overnight (16-18 hours). After overnight incubation, labelled cells were treated with DMSO or YM-254890 for the

indicated times. Fresh serum free DMEM containing 20 mM LiCl was added to each well, and the cells were incubated at 37°C and 5% $CO_2$ for 2-3 hours to let signaling generated inositol triphosphates to accumulate. After the 2-3 hour incubation, media was removed, 1 ml 0.4 M perchloric acid was added to each well, and the cells were incubated in the cold room (4℃) for 15 minutes to quench the reactions. 800 µl of perchloric acid containing supernatants were added to fresh Eppendorf tubes containing 400 µl of neutralizing buffer (0.72M KOH and 0.6M $KHCO_3$). The mixture was mixed by vortex and centrifuged at 13,000 rpm for 2 minutes. 50 µl of the supernatant was then mixed with 10 ml of scintillation fluid and used as a reference for “total labeling” for each sample. 1 ml of the supernatant was added to 3 ml of distilled water and passed through freshly prepared Dowex columns (AG1-8X; Bio-Rad). The columns were washed 2X with 2X distilled water. Bound $IP_3$ was eluted with 4 ml elution buffer (0.1 M formic acid and 1M ammonium formate). After elution, 10 ml scintillation fluid was added into each sample and counted in a liquid scintillation counter (Beckman Coulter). The eluted samples containing IP3 were divided by the reference “total labelling” sample to obtain the percent conversion of myo-inositol into $IP_3$ for each sample.

**Infection of foreskin fibroblasts** $10^5$ human foreskin fibroblasts were plated into individual wells of 12-well cell culture plates one day before infection. Human fibroblasts were infected at a MOI of 1 (i.e. $10^5$ PFU of virus per $10^5$ cells). Twenty-four hours post-infection, media were aspirated, and replaced with fresh media containing 1μCi/ml $^3$H-myoinositol and either DMSO or YM-254890. After an additional overnight incubation, the cells were used in $IP_3$ accumulation assays as described above.

**Statistical analysis.** Results are presented graphically and represent the mean ± S.E. of three or more experiments. The intensities of bands on immunoblots were quantified using Image J software. Statistical analyses were calculated with unpaired two-tailed Student's *t* tests using GraphPad Prism® 6 software. Differences were considered significant at $p < 0.05$. *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Nonsignificant changes are denoted by n.s., and represent $p > 0.05$.

## Results

### The Gαq inhibitor YM-254890 blocks $IP_3$ accumulation in cells expressing HCMV-US28 or MCMV-M33 but not in cells expressing KSHV-ORF74

Since HCMV-US28 has been shown to trigger ligand-independent signaling through Gαq using several independent methods (67), we first sought to validate the YM-254890 compound using HEK cells stably expressing US28. US28 expressing cells were treated with increasing concentrations of YM-254890 and assessed for phospholipase C β (PLCβ) activity driven by US28 (Figure 13A). The results demonstrate that YM-254890 caused a 74% reduction in PLCβ activity at a concentration of 1nM and a 90% reduction in PLCβ activity when the concentration of YM-254890 was increased to 20 nM. These data indicated that YM-254890 is a potent inhibitor of US28 dependent signaling through Gαq and confirm that US28-dependent signaling is Gαq specific.

Based on previous studies(63,67), which demonstrate that MCMV-M33 can trigger similar constitutive signaling pathways to that of US28, we hypothesized that YM-254890 would also suppress PLCβ activity triggered by M33. To that end, HEK cells were transiently transfected with M33 (and with US28 for control purposes) and treated with YM-254890 or DMSO vehicle for 24 hours, and PLCβ activity was measured as above. As predicted, M33 driven PLCβ activity was totally suppressed

when cells were pretreated with 20nM or 100 nM YM-254890 (Figure 13B). Control cells transiently

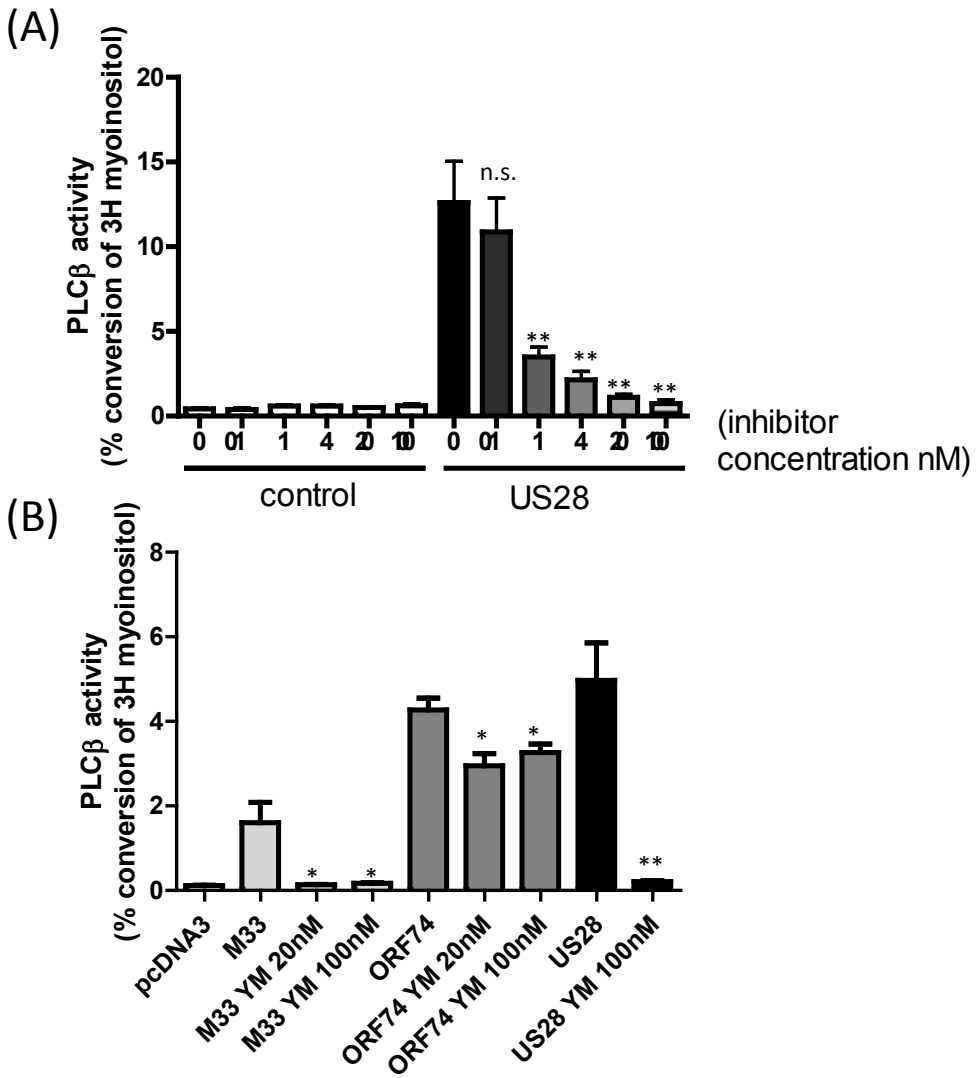



**Figure 13. YM-254890 inhibits US28 and M33 GPCR induced PLC-β signaling.**

(A) Control HEK cells and stable US28 expressing cells were labelled with 1mCi/ml $^{3}$H-myoinositol overnight, and pretreated with indicated concentration for 5 minutes before $IP_3$ accumulation assay. $IP_3$ accumulation was measured using standard techniques and converted into percent conversion of input $^{3}$H-myoinositol. The data represent five independent experiments. (B) HEK cells were transfected with pcDNA3 (vector), pcDNA3-M33flag, pcDNA3-flagORF74, or pcDNA3-flagUS28. Transfected cells were pretreated with indicated concentration of YM-254890 overnight. $IP_3$ accumulation was measured and converted into percent conversion of input $^{3}$H-myoinositol. The data represent 3 independent experiments. *:$p<0.05$, **:$p<0.01$, n.s.: not statistically significant.

expressing US28 were equally sensitive to YM-254890 confirming the results observed with US28 stable cell lines in Figure 13A.

KSHV-ORF74 is a third member of the herpesvirus vGPCR family and is also known to activate a constitutive signaling pathway leading to activation of PLC-β(65). In contrast to HCMV-US28 and MCMV-M33 that are encoded by the cytomegaloviruses, prototypical members of the β-herpesvirus family, KSHV-ORF74 is encoded by the Kaposi-sarcoma herpesvirus, a prototypic member of the γ2-herpesvirus family (202). Thus, one might expect ORF74 to function in a manner somewhat distinct from that of US28 or M33. Previous studies have shown that ORF74 can couple to multiple Gα subunits including Gαq. To determine if KSHV-ORF74 signaling is sensitive to YM-254890, HEK cells were transiently transfected with ORF74, treated with vehicle or YM-254890 and examined for PLC-β mediated $IP_3$ accumulation. Interestingly, YM-254890 caused only a modest, but statistically significant reduction (31%) of $IP_3$ , even when using the YM-254890 compound at the higher dose of 100nM (Figure 13B). Thus, while the YM-254890 compound is somewhat effective against ORF74, this effect is much weaker than that observed for US28 (96% reduction) or M33 (91% reduction) (Figure 13B). This result suggests that the PLC-β induced $IP_3$ accumulation triggered by US28 and M33 is predominantly (perhaps totally) Gαq dependent while the PLC-β induced $IP_3$

accumulation triggered by ORF74 is only partially Gαq dependent and likely to induce PLC-β dependent $IP_3$ accumulation using G-proteins other than Gαq, most likely Gαi.

**US28 and M33 but not ORF74 use similar molecular mechanisms to activate downstream signaling components**

Based on results of our $IP_3$ accumulation assays, we conclude that while US28, M33 and ORF74 all can stimulate PLC-$\beta$ activity; US28 and M33 do so by activating G$\alpha$q-dependent signaling while ORF74 does so through a mostly Gαq-independent process. In addition to the proximal effector PLC-β, US28, M33 and ORF74 each also activate the more distal signaling pathways including the transcription factors CREB (cAMP responsive element binding protein), NFAT (Nuclear Factor in activated T-cells) and NF-κB (Nuclear Factor kappa light chain binding protein). Therefore, we next wished to determine whether these vGPCRs used a Gαq-dependent or Gαq-independent signaling mechanism to activate these transcription factors. We again took advantage of the Gαq inhibitor YM-254890 to address this question. Luciferase-based reporter gene assays were utilized to measure the activation of each of the transcription factors. In US28 expressing cells, YM-254890 treatment leads to a dose dependent decrease of CREB- and NFAT-dependent reporter gene activity (60% and 52% percent respectively) (Figure 14A). In contrast US28-dependent activation of NF-kB was insensitive to the effects of YM-254890

(Figure 14A). The effects of YM-254890 on CREB or NF-AT were not as complete as that of PLC-$\beta$ (~60% versus 90%) and at this point we don't know if this is due to a difference in assay sensitivity or if the CREB/NF-AT transcription factor signaling might be somewhat independent of G$\alpha$q. In M33 expressing cells, CREB and NFAT activity were strongly reduced by 20nM YM-254890 treatment, while NF-KB activity is unaffected similar to that observed with US28 (Figure 14B). The lack of effect of YM-254890 is not unanticipated as previous results from our group has demonstrated using G$\alpha$q knockout MEFs and M33 mutants that NF-kB signaling from M33 occurs via a G-protein independent mechanism. The details underlying US28 and M33 mediated NF-κB signaling remain unclear. Lastly, we examined the effects of the YM-254890 on ORF74 mediated activation of the same transcription factors. Given that our studies had demonstrated that ORF74 signaling through the proximal effector PLC-$\beta$ was mostly independent of G$\alpha$q, we felt that we would likely see a similar lack of effect of the YM compound of ORF74 signaling to these transcription factors. As predicted YM-254890 treatment did not have influence on CREAB, NFAT, and NF-KB activity even when an excess concentration (100 nM) was used in the experiment (Figure 15). While the ORF74 mediated activation of these transcription factors is unaffected by the YM-254890 compound, these results do provide an important perspective on the activity of this compound as they enable us to

have increased confidence that the YM-254890 compound is a specific inhibitor of Gαq and M33/US28 signaling pathways rather than be a non-specific toxin to a number of pathways.

In summary, the results demonstrated that CREB and NFAT activated by US28 and M33 are at least partially Gαq dependent, while NF-KB activation by US28 and M33 is not modulated by Gαq. In contrast, although ORF74 can also activate CREB, NFAT, and NF-KB, the activation of these transcription factors by this particular vGPCR occurs via a distinct G α q independent mechanism. It is interesting that while both the β- and γ-herpesviruses have acquired GPCR homologs and appear to activate a similar set of signaling pathways, the viruses seem to have evolved to activate these pathways using distinct mechanisms.

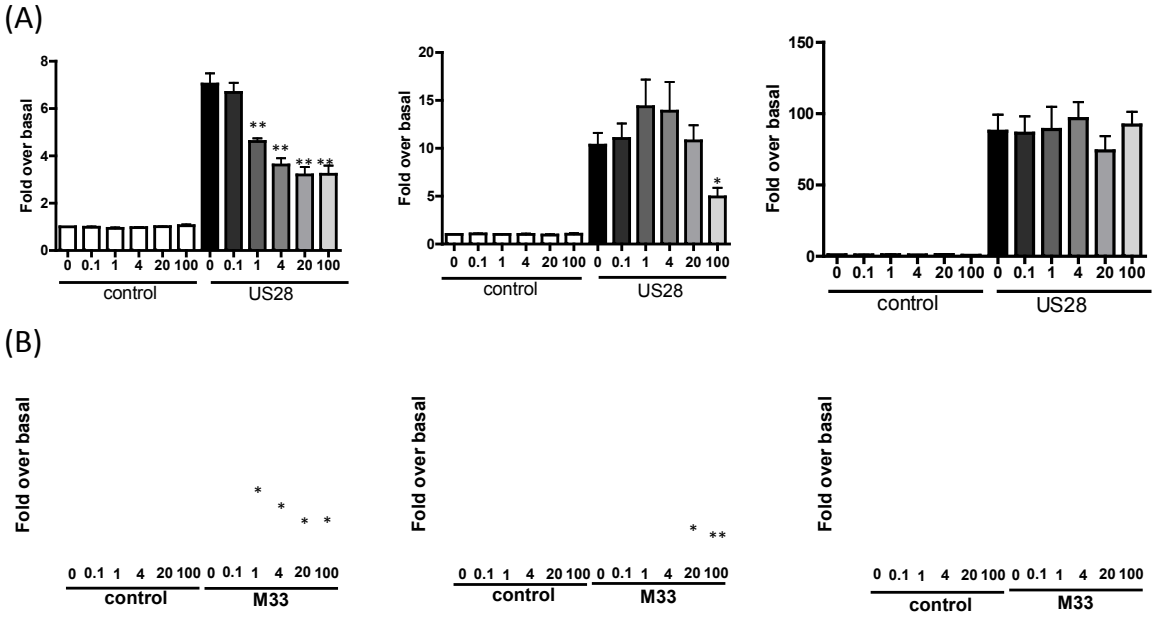



**Figure 14. YM-254890 inhibits US28 and M33 induced activation of CREB and NFAT but not NF-KB**

(A) Control HEK cells and US28 expressing cells were transfected with CREB, NFAT, and NF-kB reporter plasmids (from left to right). (B) ) Control HEK cells and M33 expressing cells were transfected with CREB, NFAT, and NF-kB reporter plasmids (from left to right). Cells were treated with vehicle (DMSO) or the indicated concentrations of YM-254890 for 48 hours. Luciferase assays were performed 48 hours after transfection. The data represent 3-4 independent experiments performed in duplicate. *:$p<0.05$, **:$p<0.01$.

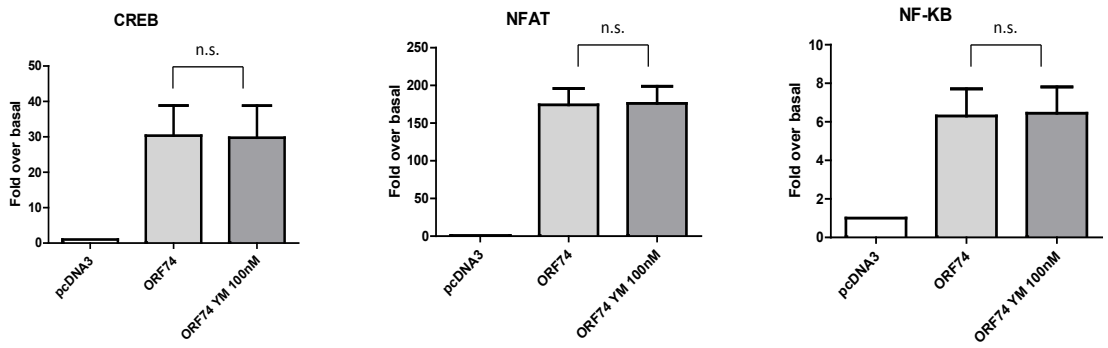



**Figure 15. YM-254890 has no effect on ORF74 induced activation of CREB, NFAT, and NF-κB.** HEK cells were transfected with the indicated reporter constructs, internal renilla control and ORF74. Luciferase reporter assays were performed. Cells were treated with vehicle or 100nM YM-254890 at the time of transfection and reporter assays were performed at 48 hours post-transfection. The ratio between firefly luciferase and renillla-luciferase obtained from vector control transfected cells (pcDNA3) was set to 1. The data represent 3 independent experiments performed in duplicate. n.s.: not statistically significant.

**YM-254890 can abolish US28-induced $IP_3$ accumulation in HCMV infected human foreskin fibroblasts**

Since YM-254890 has been shown to have potent inhibitory effects on US28 and M33 vGPCR signaling in transfected cells, the activity of YM-254890 toward vGPCR signaling in infected cells was the next goal of this research. We have previously demonstrated that infection of fibroblasts with the clinical isolate HCMV-FIX strongly induces PLC-β activity and $IP_3$ accumulation at 48 hours post-infection and that this occurs in a US28 dependent fashion as US28 null viruses lack ability to induce PLC-β activity (203). We therefore used wildtype and US28-null HCMV-FIX strains and analyzed PLC-β activity/$IP_3$ accumulation in cells with or without the YM-254890 compound. Human foreskin fibroblasts were infected with HCMV-FIX or HCMV-FIXΔUS28 strain at an MOI of 3, YM-254890 and $^3$H-myoinositol were added at 24 hours post-infection and $IP_3$ accumulation assays were performed at 48 hours post-infection to determine whether YM-254890 can inhibits PLCβ in the context of infection. While wildtype FIX virus infection caused a dramatic induction of PLCβ activity, infection of US28-null FIX virus did not lead to any induction of PLCβ activity, confirming our earlier findings that that the infection induced $IP_3$ accumulation is derived from a US28 triggered signal (203). When cells infected with wild type virus were treated with YM-254890, a dose dependent inhibition of PLCβ activity was

observed, indicating that YM-254890 can also inhibit US28 signaling in infected cells (Figure 16).

In summary our data demonstrates that YM-254890 is a specific and potent inhibitor of cytomegalovirus vGPCR signaling in both transfection and infection based conditions. The YM-254890 compound is a useful reagent as it will allow us to further dissect the signaling pathways triggered by the vGPCRs in vitro and might ultimately prove to be a novel pharmacological agent that we could use to inhibit vGPCR induced pathogenesis in vivo.

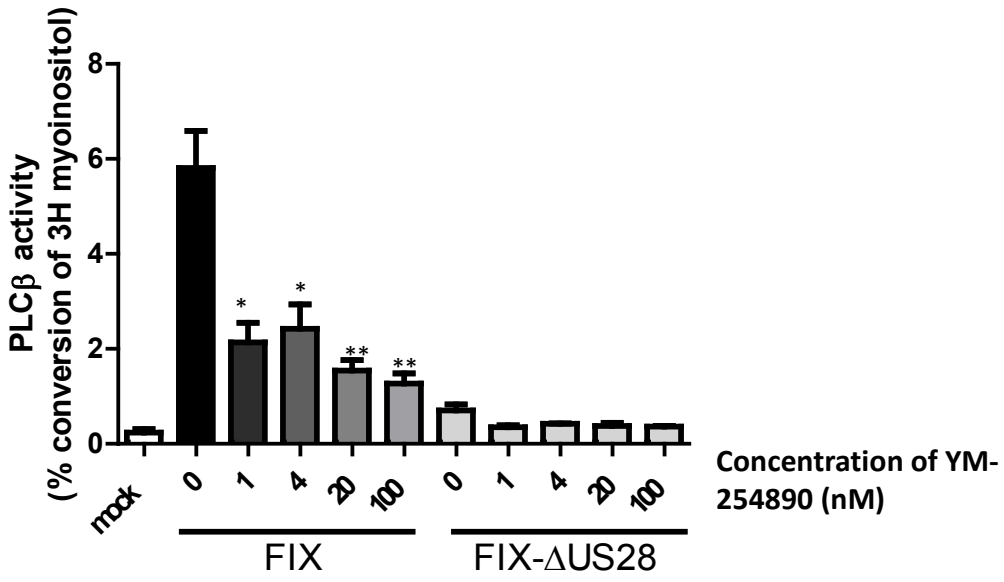



**Figure 16. YM-254890 blocks US28-induced $IP_3$ accumulation in HCMV infected human foreskin fibroblasts**

Human foreskin fibroblasts were infected with FIX and FIX-ΔUS28 virus. At day 1 post-infection, cells were treated with indicated concentration of YM-254890. $IP_3$ accumulation assays were performed at 48 hours post-infection. The data represent 3 independent experiments performed in duplicate. *$p < 0.05$; ** $p < 0.01$.

**Discussion**

YM-254890 has been shown to be a potent and specific Gαq specific inhibitor in platelets and in various mammalian cells (25,201,204,205), but this compound has not been previously used to study viral encoded GPCR signaling. We find that YM-254890 can efficiently block PLC-β/$IP_3$ signal transduction induced by the cytomegalovirus vGPCRs HCMV-US28 and MCMV-M33, but cannot block PLC-β/$IP_3$ signaling induced by the Kaposi sarcoma herpesvirus vGPCR KSHV-ORF74. Therefore the CMV and KSHV GPCRs appear to use distinct mechanisms to induce PLC-β signaling. Our data indicate that pharmacological approaches using YM-254890 are viable alternatives to genetically modified animals for the dissection of functional effects induced downstream of cytomegalovirus vGPCR mediated Gαq signaling in vivo.

Our studies indicate that US28, M33, and ORF74 all can also trigger activation of the CREB, NFAT, and NF-κB transcription factors, but that the mechanism(s) employed by these vGPCRs to activate these transcription factor signaling pathways are not the same. Pre-treatment with YM-254890 indicates that US28 and M33 both trigger CREB activation through a Gαq-dependent pathway, but that NF-κB activation occurs independently of Gαq. In the case of NFAT activation, YM-254890 inhibits US28 induced NFAT activation only at high YM-254890 concentrations, while

YM-254890 suppresses M33-driven NFAT activation even at low concentrations either suggesting that US28 driven NFAT activation is partially Gαq independent or that sustained high levels of Gαq signaling is required for NFAT activation.

Although ORF74 also triggers strong $IP_3$ accumulation, activation of the CREB, NFAT, and NF-κB transcription factors are not inhibited by YM-254890, suggesting that these signals are not initiated by Gαq. Interestingly, a recently published study demonstrated that ORF74 and US28 can activate NFAT through a PLC independent pathway (206), thus providing a plausible explanation for our data, which indicates that US28 and ORF74 mediated NFAT signaling is less sensitive or insensitive respectively to the effects of YM-254890.

In this study, we also demonstrate that YM-254890 efficiently blocks $IP_3$ accumulation induced by US28 in infected cells, and find that even overnight treatment with YM-254890 does not cause cytotoxicity in infected cells. This is an important and notable finding as it was important to demonstrate that in the context of the infection, YM-254890 would also function as a specific inhibitor of CMV vGPCR signaling.

YM-254890 was initially isolated from the culture broth of *Chromobacterium sp. QS3666* by Yamanouchi Pharmaceutical Co., Ltd.(201). This cyclic depsipeptide based compound was subsequently demonstrated to inhibit ADP-induced platelet

aggregation by blocking the P2Y1 receptor signaling pathway (201). The fermentation-related assets of YM-254890 were transferred to Astellas Pharma, Inc. and then to Taiho Pharmaceutical Co., Ltd. For unknown reasons, the company with the current rights to the compound (Taiho) is not cut currently making the compound available to researchers. A related cyclic depsipeptide, FR900359(207) isolated from the Japaneese evergreen *Ardisia Crenata*, was produced for a short time by the Institute of Pharmaceutical Biology at the University of Bonn, but is now similarly unavailable. However, due to the specificity and potency of the cyclic depsipeptide compounds with known Gαq inhibitory activity, there remains significant interest by a variety of investigators for these or related compounds. Recently chemists from Washington University, St. Louis reported the synthesis of a YM-254890 analogue (WU-07047) (208) and showed that this synthetic compound can specifically inhibit Gαq signaling although it is not quite as potent as YM-254890. Moreover, with the development of methodologies to synthesize this compound, the possibility exists that modifications could be made to the synthetic compound thus improving its potency. We are currently working with this group to test the ability of the synthetic WU-07047 compound to inhibit US28 and M33 signaling.

In conclusion, we find that YM-254890 is a useful and specific Gαq specific inhibitor that could be used to study the functional consequences and mechanism(s)

of signaling induced by the cytomegalovirus vGPCRs. YM-254890 (or most likely, the recently synthesized YM-254890 analogs) will be especially useful in cell types refractory to gene silencing approaches or when the need arises to test the function of cytomegalovirus vGPCR Gαq signaling in various tissues or in a high throughput manner.

# Chapter IV

# The HCMV US28 vGPCR induces potent Gαq/PLC-β signaling in THP-1 monocytes leading to altered cellular migration and adhesion

Shu-en Wu and William E. Miller

***In preparation for publication***

**Abstract**

US28 transcripts have been detected in latently infected THP-1 monocytes but US28 protein expression has not yet been examined in either latently infected THP-1 cells or in primary monocytes/macrophages. Moreover, while US28 has been demonstrated to be a potent signaling molecule in numerous cellular systems, mechanistic information regarding US28 signaling and function in monocytes remains unknown. In this study, we show that US28 protein is ~~robustly~~ expressed in latently infected THP-1 cells, and that ectopic expression of US28 can constitutively trigger Gαq dependent signaling in THP-1 cells. This US28 signaling dramatically blocks chemokinesis through a Gαq dependent pathway while simultaneously promoting endothelial cell adhesion. It is known that monocytes are important carriers of latent HCMV and are believed to play important roles in viral dissemination. Our studies, which demonstrate that US28-driven Gαq signaling has profound effects on monocyte biology, suggest that US28 driven phenotypic changes in HCMV infected monocytes may play important roles in HCMV dissemination and/or pathogenesis.

**Introduction**

US28 is a HCMV encoded GPCR, which is structurally similar to human CC chemokine receptors, most notably, CCR-1 (181). The HCMV genome encodes four such GPCRs (US27, US28, UL33 and UL78), and of these four, US28 appears to be the most potent signaler and have the most significant phenotypic effects on infected cells (50). Although not essential for replication in vitro(182), previous studies have shown that US28 can promote migration of vascular smooth muscle cells (79), function as a chemokine sink to reduce chemokine availability in the milieu surrounding infected cells (59,182), and facilitate cell to cell viral transmission in epithelial cells (209). In human foreskin fibroblasts, the US28 protein is expressed with early to late phase kinetics (203). In myeloid cells that support the HCMV dormant or “latent” phase, US28 transcripts have been demonstrated to be expressed either transiently or persistently after infection depending on the cell type used for the experiment(27,28,72). Although US28 is thought to be one of a subset of viral genes expressed in the latent phase, what role, if any, US28 plays during latency remains unknown. In addition, since the presence of transcripts may not necessarily reflect protein expression, whether or not US28 protein is expressed and present in latently or lytically infected myeloid cells remains an interesting and important open question.

Previous results from our lab have shown that US28 triggers constitutive

signaling by coupling to Gαq in human foreskin fibroblasts, endothelial cells, vascular smooth muscle cells, and glioblastoma derived tumor cells (64). In the canonical Gαq signaling pathway, Gαq can activate phospholipase C-β to induce inositol triphosphate (IP3) accumulation, which leads to the release of calcium from the endoplasmic reticulum (ER) and the activation of protein kinases such as Protein Kinase C (PKC)(210). In addition to Gαq, other Gα subunits including Gα12, Gα13, Gα16, and Gαi have been shown to be involved in US28-dependent constitutive and/or ligand-dependent signaling (57,69,176,211). However, whether or not US28 triggers a similar or distinct set of signaling pathways in monocytes remains unexplored. Thereby, in this research we sought to examine whether US28 can trigger constitutive signals in a monocytic cell line, and if so, determine what Gα subunit is used by US28 to activate signaling. Pharmacological inhibitors have been widely used to assess G-protein signaling activity and many such inhibitors are available including Pertussis toxin (Gαi inhibitor) (212), YM-254890 (Gαq inhibitor) (25), U-73122 (phospholipase C inhibitor) (213), and Ro-32-0432 (PKC inhibitor) (214) all of which could be used to tease out the signal mechanism(s) used by US28 in monocytes.

US28 is a seven-membrane spanning protein with an extracellular amino terminus and an intracellular carboxy terminal tail (215). US28, like most members of the GPCR superfamily contains a “DRY box” motif (aspartate-arginine-tyrosine)

located in second intracellular loop at residues 128-130 that is essential for G protein coupling (216), and replacement of arginine 129 with alanine (R129A) abolishes G protein coupling (217). In addition, amino acids between residues 11 and 16 in the amino terminus of US28 are required for ligand binding(175), and deletion of residues 2 through 16 (ΔN) eliminates all known chemokine binding to US28 (203). US28 mutants such as US28-R129A and US28-ΔN are useful tools that can be used to dissect and analyze the signals, functions, and mechanisms of US28 action within cells.

The monocyte is not only a cell type in which HCMV can establish latency but also is believed to play an important role in viral dissemination. Cell migration is a crucial factor for viral dissemination, and previous studies have shown that the binding of MCP-1 or RANTES to US28 promotes vascular smooth muscle cell migration (79), while binding of Fractalkine to US28 modestly enhances macrophage migration in a rat macrophage model (58). Thus, it appears that chemokine binding to US28 can have distinct effects on cell migration in a cell type dependent fashion. It remains possible and unexplored whether or not US28 can also affect monocyte migration, a potentially important phenomenon as it is the monocyte rather than the macrophage that is the predominant myeloid cell in the blood where virus dissemination would be primarily occurring(80). In addition to cellular migration and chemotaxis, monocyte

adhesion to the endothelium is another essential element believed to be important for HCMV dissemination (218). It is known that HCMV infected monocytes display increased monocyte/endothelial cell adhesion(81), but whether US28 can influence monocyte adhesion to the endothelium remains unknown.

In this study, we found that the US28 protein is expressed in HCMV infected THP-1 monocytes, and that US28 can constitutively couple to Gαq to activate phospholipase C-β (PLC-β). In addition, we demonstrate that the US28 triggered Gαq signal inhibits THP-1 chemokinesis (basal cell migration) and promotes adhesion to endothelial cells via a Gαq→PLC-β→PKC signaling axis. The results from these studies suggest that US28 protein expression and signaling may influence the biology of latently infected monocytes thereby facilitating hematogenous dissemination of virus.

**Material and methods**

**General reagents.** Anti-human CD36 antibodies were purchased from eBioscience. Anti-FLAG M2 agarose beads and anti-FLAG M2 antibody conjugated to biotin were obtained from Sigma-Aldrich. Streptavidin conjugated to HRP was purchased from BD Pharmagen. Anti-UL44 antibody is a gift from John D. Shanley; University of Connecticut.

**Construction of US28 lentiviral mutant constructs.** pcDNA3-US28FLAG, and pcDNA3-FLAG US28 R129A were used as templates for construction of US28 lentivirus expression constructs. Primers for amplification of wildtype US28 (US28-WT): Forward primer: GTTCGGACTAGTGCCACCATGACACCGACGACGCGACCGCG; Reverse primer: GTTCGGTCTAGATTACTTGTCATCATCGTCCTTGTAGTC. Primers for amplification of amino terminal truncated US28 (US28-ΔN): Forward primer: GTTCGGACTAGTGCCACCATGGACGATGAAGCGACTCCCTGTGTC; Reverse primer is the same as reverse primer for wildtype PCR reaction. Primers for amplification of US28 containing an Arg to Ala mutation at amino acids 129 (US28-R129A) were the same as for wildtype US28. PCR reactions were performed for 30 cycles with denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds,

and extension at 72°C for 1 minute using Phusion High –Fidelity DNA polymerase (New England BioLabs Inc.). The fragments were gel purified using GeneJET Gel Extraction Kit (Thermo Scientific), and were digested with SpeI and XbaI. Digested fragments were inserted into the SpeI and XbaI sites of the pEN-TmiRC3 entry vector (provided by Iain Fraser via Addgene). Inserts in pEN vector were transferred to pSLIK-Venus (provided by Iain Fraser via Addgene) using the Gateway LR Clonase II Enzyme (Invitrogen). All constructs were verified by DNA sequencing.

**Reverse transcription PCR.** Infected THP-1 cells or peripheral blood monocytes were harvested, and RNA was isolated using RNA-Bee (Tel-Test, Inc.). 1µg of RNA was converted to complementary DNA using an iScript cDNA Synthesis Kit (Bio-Rad). PCR reactions were performed for 30 cycles with Taq DNA polymerase using similar conditions to those described above. Primers for US28 PCR are WEM-116 (forward primer) and WEM-178 (reverse primer).

**Production of Lentivirus particles for gene transduction**

4 plates of $2x10^6$ 293T cells were transfected with 3 µg of pSLIK-Venus lentiviral backbone containing US28 and US28 mutants, 1.5 µg of psPAX2, and 0.5 µg of pVSVg using a 4:1 ratio of Mirus LT-1 transfection reagent according to the

manufacturer's and cultured in DMEM supplemented with 10% FBS, penicillin and streptomycin. 24 hours after transfection, media were removed and cells were fed with fresh media. Culture media containing lentiviruses were then harvested at 48 and 72 hours post transfection. Viruses were concentrated by ultracentrifugation at 25,000 RPM for 90 minutes at 4°C. Supernatants were aspirated, and viral pellets were resuspended in 2 ml RPMI-1640 with 10% FBS and penicillin/streptomycin. 2ml of viral supernatant was used to infect $4x10^5$ THP-1 cells via spin-fection (centrifugation at 1000xg for 90 minutes at 37°C). After transduction, THP-1 cells were cultured overnight, and media were changed with fresh media in the morning. At day 2 and day 4 after transduction, THP-1 cells were analyzed by flow cytometry to examine the transduction efficiency.

**Isolation of peripheral blood monocytes.** Peripheral blood mononuclear cells were isolated using Ficoll-Paque PLUS as described previously. Once the buffy coat was isolated and washed, cells were cultured in serum free RPMI-1640 for an hour to allow monocytes to attach to the plate. Then, unattached cells were washed away with DPBS. Fresh RPMI-1640 supplemented with 20% FBS, penicillin, and streptomycin was used to maintain monocyte culture.

**Propagation and purification of HCMV virus.** The HCMV TB40E-mCherry(3XFLAGUS28) virus was generously provided by Dr. Christine O' Connor from the Cleveland Clinic (64,73). This virus was characterized and demonstrated to grow with similar kinetics and to similar titers as does HCMV TB40E. To propagate virus, HFFs were infected with TB40E viruses at an m.o.i. of 0.04. Viral supernatant was harvested at days 9, 11, and 13 post-infection. Cell Culture supernatant containing virus was centrifuged at 1800xg for 3 minutes at 21°C to remove cellular debris. The clarified supernatant was overlaid on a 20% D-sorbitol/1mM $MgCl_2$ cushion and subjected to ultracentrifugation at 24,000 rpm for 1hr at 21°C. Supernatant was decanted, and the viral pellet was resuspended in RPMI-1640 culture media. Viral supernatant was aliquoted and stored at -80°C. UV-inactivated virus was processed by shining the viral supernatant with UV in cell culture hood for 15-20 minutes.

**HCMV infection of THP-1 cells and peripheral blood monocytes.** THP-1 cells were infected with HCMV TB40E-mCherry (3XFLAGUS28) at MOIs as indicated in the figure legends. After the viral supernatant was added, cells were centrifuged at 21°C and 1000xg for 30 minutes to enhance infectivity. After overnight culture, cells were spun down and the inoculums were removed. Cells were incubated in 1X trypsin

for 5 minutes to remove attached but un-internalized virions (120). The trypsin reactions were neutralized by adding equal volumes of fresh culture media. The supernatant is aspirated, and cells were resuspended in fresh culture media. After overnight culture, culture media were removed and cells were washed with 1X PBS, and fresh media were added to the cells. UV-inactivated virus was processed by shining the viral supernatant with UV in cell culture hood for 15-20 minutes. TB-40 E mcherry US28 3xflag virus and TB-40E US28 null virus were used to infected peripheral blood monocytes with m.o.i. of 10. One day after infection, cells were washed with DPBS and resuspended in fresh RPMI-1640 supplemented with 20% FBS, penicillin and streptomycin.

**Cell culture.** Human endothelial cell line HECV was cultured in DMEM (Dulbecco Modified Eagle's Medium) supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% $CO_2$. Cells are passaged every 3 days. THP-1 monocytic cell line was maintained in RPMI-1640 (Roswell Park Memorial Institute Institute-1640) supplemented with 10% FBS, 100 IU/ml penicillin and 100g/ml streptomycin at 37°C in 5% $CO_2$. Cells were passaged every 3 days to keep the cell density below $10^6$ cells /ml.

**$IP_3$ accumulation assays.** 2X$10^5$ cells were plated into each well in 12-well plates in inositol free RPMI-1640 (Roswell Park Memorial Institute Institute-1640) supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin and 2 µ Curi of $^3$H-myoinositol for 24 hours at 37°C in 5% $CO_2$. 1 µg of doxycyclin or vehicle was added to each well to induce gene expression for another 24 hours. Cells were harvested and washed with serum free RPMI once. THP-1 cells were resuspended in 1 ml serum free RPMI supplemented with 20 mM LiCl and incubated at 37°C in 5% $CO_2$ for 2 hours. Cells were pelleted by centrifugation at 500 x g for 5 minutes. Supernatant was removed. Cells were resuspended in 1 ml percholic acid and incubated at 4°C for 15 minutes. Cells were pelleted and 800µl of supernatant was added to fresh Eppendorf containing 400 µl of neutralizing buffer (0.72M KOH and 0.6M $KHCO_3$). The mixture was mixed by vortex and centrifuged at 13,000 rpm for 2 minutes. 50 µl of the supernatant was then mixed with 10 ml of scintillation fluid and used as a reference for "total labeling" for each sample. 1 ml of the supernatant was added to 3 ml of distilled water and passed through freshly prepared Dowex columns (AG1-8X; Bio-Rad). The columns were washed 2X with 2X distilled water. Bound $IP_3$ was eluted with 4 ml elution buffer (0.1 M formic acid and 1M ammonium formate). After elution, 10 ml scintillation fluid was added into each sample and counted in a liquid scintillation counter (Beckman Coulter). The eluted samples containing IP3

were divided by the reference “total labelling” sample to obtain the percent conversion of myo-inositol into $IP_3$ for each sample.

**Immunoprecipitation and western bloting.** Cells infected with TB-40E mcherry 3X flag virus were harvested at indicated days post infection in RIPA buffer. THP-1 control cells and THP-1 cells expressing US28 and US28 mutants were harvested in RIPA buffer. Cell lysate were passed through a 22 G x 1 ½ needle for ten times, and centrifuged to remove cell debris. 50 µl of cell lysate was saved as whole cell lysate. 20 µl of M2-aggarose beads were added to the rest of the cell lysate, and samples were incubated at 4°C overnight. M2-agarose beads were pelleted by centrifugation and supernatant were aspirated. Agarose beads were washed 3 times with RIPA buffer. 50µl of 3X sample buffer was added to each sample, and let the sample stay in room temperature for 30 minutes. 20 µl of each immunoprecipitation sample and whole cell lysate were used for 10% SDS-PAGE electrophoresis. After electrophoresis, proteins were transferred from SDS-PAGE to nitrocellulose through semi-dry transfer apparatus. The cellulose membrane was incubated in 5% skim milk for 1 hour. Then the membrane was incubated in rabbit polyclonal anti-flag antibody (1: 1000 dilution) at 4°C overnight. The membrane was washed 3 times with TBST, and was incubated with goat anti-rabbit HRP conjugated secondary antibody (1:2000

dilution) for 3 hours. The membrane was washed 3 times with TBST, and subjected to antibody detection using the SuperSignal West Pico or Fento chemiluminescent substrate (Thermo Scientific). Luminescence emitted from the membranes was detected by classic blue autoradiography film BX. Films were developed by Kodak min-R mammography processor.

**Transwell migration assays.** $1.5x10^5$ THP-1 cells were added into each 5 μm pore size insert (Costar), and 600 μl of RPMI-1640 culture medium containing MCP-1 (10 nM) or vehicle was added to bottom chamber. Cells were allowed to migrate for 2 hours at 37°C, 5% $CO_2$. Cells migrating to the bottom chamber were collected, and resuspended in 200μl RMPI-1640 culture medium. Input cells were also resuspended in 200 μl culture medium. The number of cell migrating to the bottom chamber and the number of input cells were determined by flow cytometry. The ratio between the cells migrating to the bottom chamber and the input cell was converted into percentage.

**Monocyte-endothelium adhesion assay.** $10^5$ HECV endothelial cells were plated into each well in a 12 well plate and cultured overnight. About 500,000 THP-1 cells were harvested and plated into each well to co-culture for 1 hour. The suspended cells were removed and each well was washed twice 2 times with culture media. Adherent

cells were trypsinized to detach them from the plate. Cells were collected and stained with anti-human CD36 antibody for an hour. Cells were washed once with DPBS. Cells were resuspnded in 200 µl DPBS. Cells from each well were count by flow cytometry, and the number of total cells and CD36 positive cells (THP-1) are measured by flow cytometry. The number of CD36 positive cells was divided by the total number of THP-1 cells put into each well and the ratio was converted to percentage.

**Collagen binding assay.** 24-well plates were coated with 0.25 mg/ml collagen dissolved in 0.2% acetic acid/DPBS for 15 minutes. Plates were washed with DPBS twice. $5x10^5$ cells were plated into each well and incubated for 1 hour at 37 °C 5% $CO_2$. After incubation, each well was washed with culture media twice. Cells adherent to plate were trypsinized for 5 minutes, and trypsin was neutralized by adding an equal amount (100 µl) of culture medium. The number of cells adherent to the plate was counted by flow cytometry. The number of input cell in each well was also determined by flow cytometry. The percentage of cells adherent to the plate was determined by dividing the number of cells adherent to the plate by the number of the input cell in each well.

**Statistical analysis.** Results are presented graphically and represent the mean ± S.E. of three or more experiments. The intensities of bands on immunoblots were quantified using Image J software. Statistical analyses were calculated with unpaired two-tailed Student's *t* tests using GraphPad Prism® 6 software. Differences were considered significant at $p < 0.05$. *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Nonsignificant changes are denoted by n.s., and represent $p > 0.05$.

## Results

### Examination of US28 RNA and protein expression in peripheral blood monocytes and in the THP-1 monocytic cell line.

While previous studies have indicated that US28 transcripts can be detected in THP-1 monocytes and in primary myeloid cells (26-28), it remained unclear if the US28 protein was indeed expressed in any of these settings. We initiated our studies by first infecting THP-1 cells with a recombinant HCMV TB-40E strain containing an epitope tagged US28 gene (TB40E-US28 3XFLAG) and re-examined US28 expression by RT-PCR. TB-40E is a HCMV strain with enhanced tropism for endothelial and myeloid cells (74,77), and compared to laboratory adapted strains such as AD169 and Towne, TB-40E has a more complete complement of viral genes including genes in the ULb' region of the viral genome (76). Infected cells were harvested at various time points

post-infection, and RNA was isolated for use in RT-PCR. US28 transcripts were detected from 1-8 days post-infection in THP-1 cells (Figure 17A). We also examined US28 mRNA levels in peripheral blood monocytes derived by briefly attaching PBMCs to plastic and similarly find that US28 mRNA is expressed in these cells, confirming that US28 is transcribed in HCMV infected monocytes (Figure 17B).

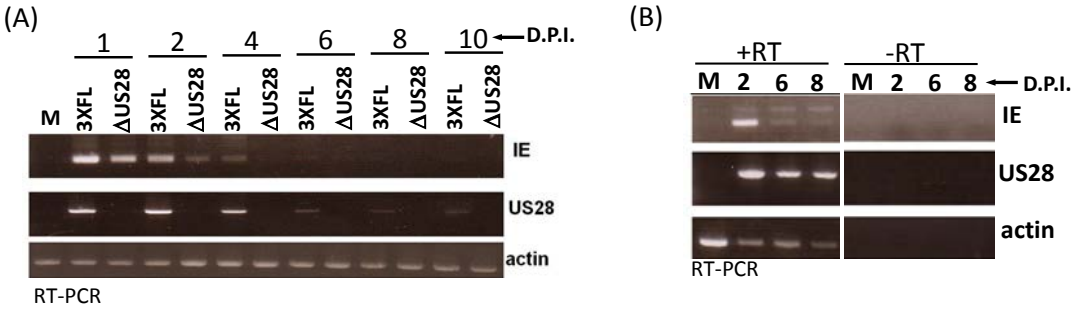



**Figure 17. US28 RNA is expressed in infected THP-1 cells and in peripheral blood derived monocytes**

(A) THP-1 cells were infected with TB-40E virus or TB-40E US28-null virus at a MOI of 10. Cells were harvested at day1,2, 4 , 6,8 and 10 post-infection. RNA samples were converted into cDNA by reverse transcription. PCR was then performed to analyze the expression of HCMV immediate-early genes (IE), US28 (US28) and cellular actin as an internal control (actin). (B) Peripheral blood monocytes were isolated and infected with TB-40E virus. Infected cells were harvested at days 2, 6, and 8 post infection. RNA samples were converted into cDNA by reverse transcription and PCR was then performed to analyze gene expression as described above. M: mock infection, d.p.i: day post infection.

We the sought to examine whether US28 protein is expressed in HCMV infected THP-1 cells. To address this point we infected THP-1 cells with wildtype and UV-inactivated HCMV strain TB40E-US28 3X FLAG. Since UV-inactivated virus

infects cells but does not initiate viral gene expression, it serves as an appropriate control to tell us whether US28 protein is present in infected THP-1 cells and determine whether this US28 protein is the result of *de novo* protein synthesis or delivered into the cell by infecting viral particle. This latter part of the experiment was included in our studies as several groups have proposed that US28 present in the viral particles could be inserted into the plasma membrane of the infected cells and initiate signaling beneficial to viral replication (219,220). FLAG-tagged US28 protein was immunoprecipitated with anti-FLAG M2 affinity gel and analyzed by western blotting using biotinylated anti-FLAG M2 antibodies. Our results demonstrate that US28 protein is detectable in the THP-1 cells infected with live but not UV-inactivated virus. US28 protein expression is delayed somewhat relative to immediate early IE1/IE2 proteins but is clearly detectable at 2 and 4 days post-infection virus (Figure 18). Interestingly, consistent with the observation that THP-1 cells do not support efficient lytic replication (136,221), the delayed-early UL44 protein is not detectable in this experiment. These results indicated that US28 protein is expressed in non-permissively infected THP-1 cells and the presence of US28 protein is through *de novo* protein synthesis rather than being delivered by the infecting virus particle.

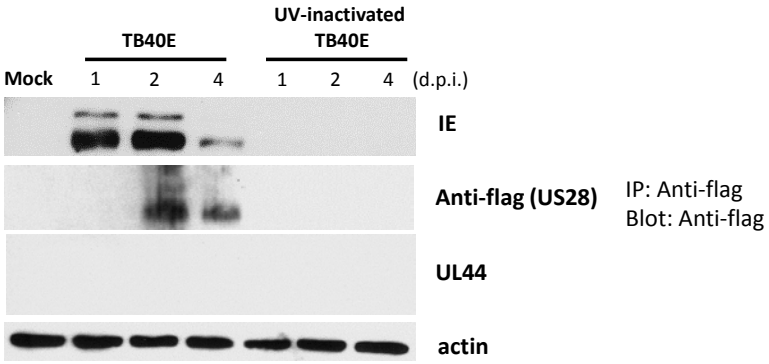



**Figure 18. US28 protein is expressed in infected THP-1 cells**

THP-1 cells were infected with TB-40E virus or UV-inactivated virus at MOI of 10. Cells were harvested at days 1, 2, and 4 post-infection. Whole cell lysates were analyzed by western blot to examine HCMV immediate early (IE) protein, UL44, and actin (internal control). Immunoprecipitation followed by western blotting with anti-flag antibodies were performed to analyze US28 protein expression. d.p.i: day post infection.

## US28 triggers constitutive Gαq dependent signaling pathways in THP-1 monocytes

While several previous studies have shown that US28 can constitutively activate Gαq and induce PLC-β dependent $IP_3$ accumulation in fibroblasts, smooth muscle cells, and endothelial cells (64,203), whether US28 can trigger constitutive signaling in myeloid cells such as monocytes remains unknown. In addition, due to the low levels of US28 protein expressed during latent infection and the possibility that unknown feedback desensitization pathways could affect US28 signaling in the context of a latent infection, we developed an inducible protein expression system to examine US28 triggered signaling in THP-1 monocytes.

Our approach involved the introduction of a lentivirus, pSLIK-VENUS, carrying

doxycycline/tetracycline inducible US28 expression cassettes into THP-1 cells. Immunoprecipitation and western blot techniques (described above) were then used to confirm US28 expression. Using this system, we find that US28 protein is tightly repressed in the absence of doxycycline (DOX), but robustly induced within 24 hours after addition of DOX to the media (Figure 19).

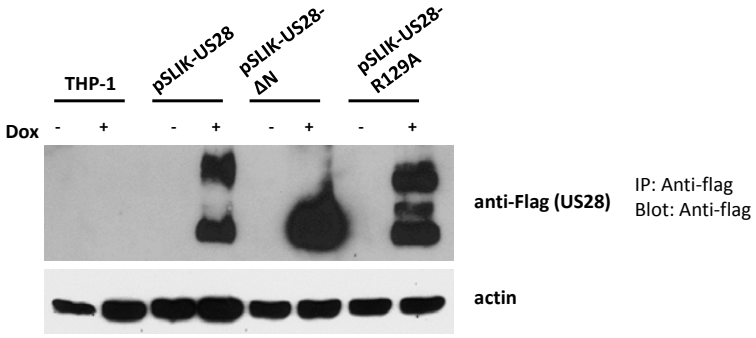



**Figure 19. Expression of US28 and US28 mutant proteins in stably transduced THP-1 cells.** THP-1 cells were transduced with Tet-regulatable pSLIK based lentivruses and sorted on the basis of expression of the Venus marker protein. Sorted cells were treated with 1μg/ml of doxycycline for 24 hours to induce US28 expression. Immunoprecipitation and western blot with anti-flag antibody were used to detect US28 and mutant protein expression. Whole cell lysates were analyzed by western blot to detect actin expression.

This newly developed, tightly regulatable myeloid-specific US28 expression system provides a robust model to explore US28 signaling as it affords us with the ability to direct US28 expression only when desirable and alleviates any problems that could be attributed to chronic long-term expression of this highly active viral protein.

To examine the effects of US28 on Gαq/PLC-β driven $IP_3$ accumulation, THP-1 cells were labeled with $^3$H-myoinisitol for 48 hours, and US28 expression was induced

with 1μg/ml DOX for the final 24 hours of the labeling protocol. The results of $IP_3$ accumulation assays demonstrated that US28 causes a 3-fold increase in $IP_3$ accumulation compared to control THP-1 cells or US28 cells without DOX (Figure 20). Since $IP_3$ accumulation is an indicator of the activated of phospholipase C-β(222), a downstream effector of Gαq, these result suggested that US28 can constitutively activate Gαq in THP-1 cells. To verify that the $IP_3$ signaling is triggered by US28 coupling to G proteins, we used the US28 DRY box mutant US28 R129A, which cannot couple to heterotrimeric G proteins (217). Expression of US28 R129A was confirmed by immunoprecipitation and western blot and shown to be similar to that of wildtype US28 (Figure 19). While the protein expression levels of US28 R129A are similar to that of wildtype US28 after doxycycline treatment, $IP_3$ signaling is not triggered by US28 R129A mutant (Figure 20), indicating that the US28-driven $IP_3$ accumulation is indeed G protein-dependent.

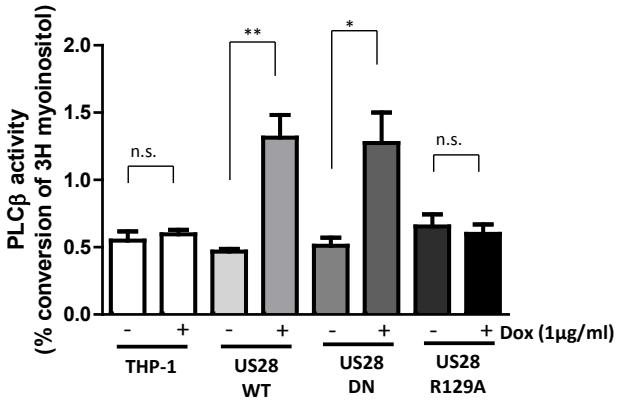



**Figure 20. US28 induces $IP_3$ accumulation in THP-1 cells**

Transduced THP-1 cells were labeled with $^3$H-myoinositol overnight and then US28 expression was induced with 1 µg/ml of doxycycline or vehicle ($H_2O$) for an additional 24 hours. Assays were performed as described previously. The percentage conversion of $^3$H-myoinositol into $^3$H-$IP_3$ conversion was calculated by dividing inositol phosphates eluted from anion exchange columns by total label counts. The graph represented four independent experiments. Unpaired Student t tests were used to examine the data for statistical significance. DN: N-terminal deletion US28, R129A: US28 with DRY box mutation**:$p<0.01$, *: $p<0.05$, n.s.: not statistically significant.

Since it is known that US28 can bind to multiple chemokines(175,215), it is possible that chemokines secreted by monocytes such as MCP-1(223,224) could be binding to US28 and activating signaling in an autocrine fashion. Therefore the US28 N-terminal truncated mutant (DN US28), which lacks amino acid 2 to 16 and cannot bind to chemokines(203) was used to determine whether the $IP_3$ accumulation induced by US28 is occurring a constitutive or ligand dependent signaling process. The expression of DN US28 was also verified by immunoprecipitation and western blot (Figure 19), and the expression levels were found to be similar to both US28 wildtype and US28 R129A. THP-1 cells expressing DN US28 showed a significant induction of

$IP_3$ accumulation when compared to control cells or DN US28 cells without DOX, indicating that the US28 signaling we observe is due to constitutive signaling activity and unlikely to involve autocrine signaling mediated by secreted chemokines (Figure 20). Taken together, the data obtained from these experiments indicated that US28 can constitutively couple to heterotrimeric G proteins to kindle signal transduction in monocytes.

While the cellular chemokine receptors mostly highly similar to US28 are classically coupled to Gαi (173,174), much of the previous work with US28 has demonstrated that it is constitutively coupled to Gαq. Therefore, to determine which Gα subunit is utilized by US28 for this constitutive signaling in monocytes, we re-examined US28 induced $IP_3$ accumulation using pharmacological inhibitors of the relevant G-proteins. In particular, Gαi and Gαq inhibitors were used along with a PLC-specific inhibitor to examine this question. YM-254890, a Gαq specific inhibitor(25), suppressed the $IP_3$ signaling induced by US28, as did U-73122, a PLC inhibitor (Figure 21)(213). However, pertussis toxin, a Gαi inhibitor(212), had no effect on $IP_3$ accumulation activated by US28 indicating that in the case of US28, Gαq and not Gαi is the specific G-protein responsible for activating this signaling (Figure 21). In summary, using a combination of US28 mutants and pharmacological inhibitors, we demonstrate that US28 can constitutively trigger Gαq-dependent signaling

transduction in monocytes.

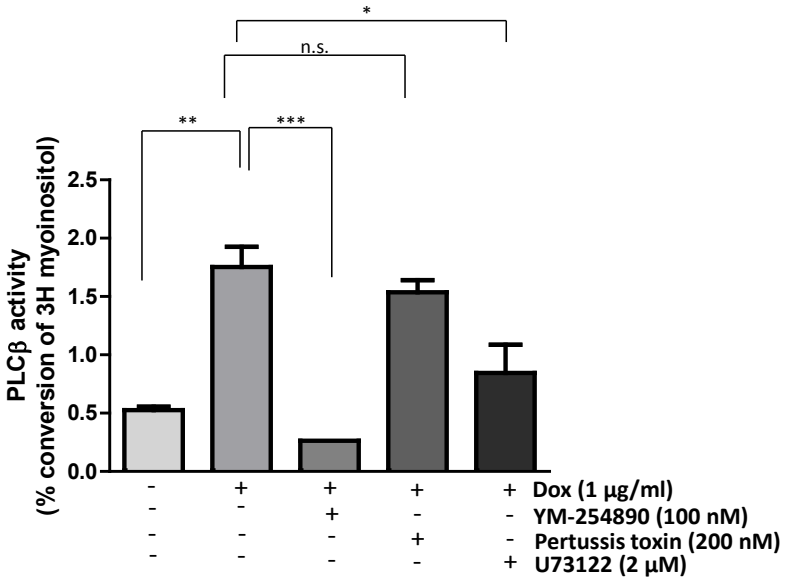



**Figure 21. Inhibition of US28 signals by Gαq and PLCβ inhibitors** Transduced THP-1 cells were labeled with $^{3}$H-myoinositol 1 day before induction, and treated with 1 µg/ml of doxycycline or vehicle ($H_2O$) for 24 hours. THP-1 cells were pretreated with inhibitors of Gαq (YM-254890, 100 nM), PLCβ (U73122, 2 µM ), or Gαi (pertussis toxin, 200nM) for 30 minutes before $IP_3$ assays. Percent conversion of IP3 was calculated as described previously. The graph represents three independent experiments performed in duplicate. Unpaired Student T tests were used to analyze the data. YM: YM-254890, PTX: pertussis toxin, ***: p=0.001,**:p<0.01, *: p<0.05, n.s.: not statistically significant.

**US28 inhibits THP-1 monocyte chemokinesis**

While US28 protein is expressed in HCMV infected THP-1 monocytes, the function that US28 plays in this cell type remains unknown. Our heterologous expression experiments indicate that US28 is a robust activator of G-protein dependent signaling suggesting that this signaling activity may impart significant phenotypic changes to these cells. Since it has been reported that US28 affects migration of smooth muscle cells and rat macrophages(58,79), we wished to explore

what effect, if any, US28 signaling has on migration of monocytes using our system. To explore this question, Boyden-chamber transwell migration assays were employed. In these assays spontaneous movement of cells across the transwell membrane into the bottom chamber is defined as chemo- “kinesis” while chemokine dependent movement of cells into the bottom chamber is defined as chemo-“taxis”. Interestingly, when US28 expression is induced overnight following addition of DOX, this severely inhibited spontaneous chemokinesis of THP-1 monocytes (Figure 22A). In other words, THP-1 monocytes expressing US28 are much less motile than the control cells

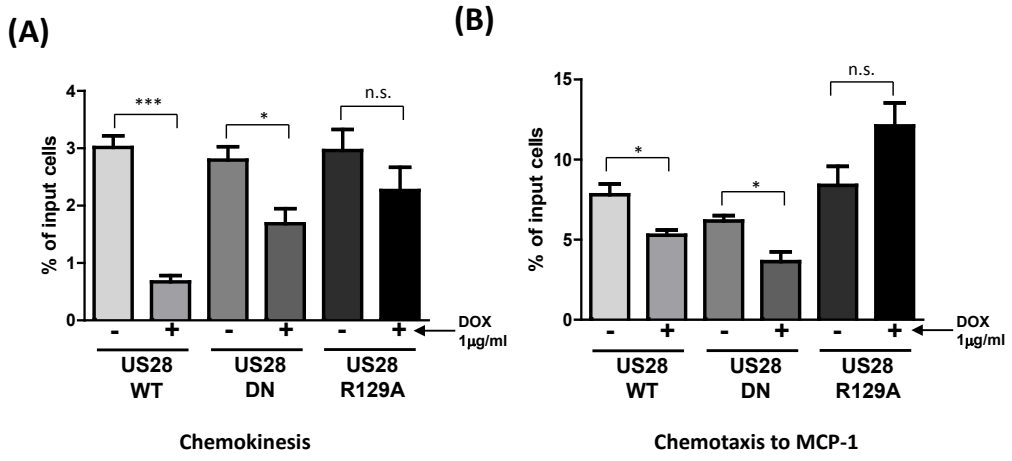



**Figure 22. US28 derived inhibitory effects on THP-1 chemokinesis and chemotaxis.**
(A) Transduced THP-1 cells were treated with 1 µg/ml of doxycycline or vehicle ($H_2O$) for 24 hours to induce US28 expression. Transwell assays were performed for 2 hours in the absence of chemokines. (B) Transduced THP-1 cells were treated with 1 µg/ml of doxycycline or vehicle ($H_2O$) for 24 hours to induce US28 expression. Transwell assays were performed in the presence of 10 nM of the CC chemokine monocyte chemotaxis protein 1 (MCP-1). Numbers of input cells and cells migrating to the bottom chamber were determined by flow cytometry. The ratios between cells in the bottom chamber and input cells were converted into percent migration. The graphs represented four independent experiments. Unpaired Student t test was used to analyze the data. ***: $p<0.0001$, *: $p<0.05$, n.s.: not statistically significant.

lacking US28 expression.

We then tested the effect of the monocyte chemotactic protein MCP-1 on THP-1 monocyte migration. In this experimental setting the effects of MCP-1 could be two fold. First, it is a potent chemotactic agent towards monocytes and second, it has the potential to bind US28 and alter its signaling properties. MCP-1 induced chemotaxis to the bottom chamber was only modestly decreased in cells expressing US28 (Figrue 22B) indicating that while US28 expression paralyzes basal cell migration (chemokinesis), they retain the ability to migrate towards chemoattractants suggesting that US28 expression may reprogram the monocytes to migrate specifically to a subset of chemokines.

Our results indicated that US28 can inhibit basal migration (chemokinesis) and possibly de-regulate chemokine-driven migration (chemotaxis) of THP-1 cells. We wished to further explore this phenomenon using our signaling dead (R129A) and chemokine-binding defective (DN) US28 mutants. Based on our signaling studies, we know that both US28 and DN US28 can initiate constitutive $G\alpha q \rightarrow IP_3$ signaling, while US28 R129A cannot. Therefore, using these mutants we can determine whether the inhibitory effect of US28 on cell migration is signaling dependent and/or chemokine binding dependent. The effect of US28 on inhibiting chemokinesis is lost in cells expressing US28 R129A but not in cells expressing DN US28, indicating that US28

derived signaling is responsible for the inhibition of cell migration (Figures 22 A and B).

Based on our findings that constitutive US28 signaling inhibits chemokinesis, we next wished to investigate the molecular details of the signaling pathway involved in inhibition of chemokinesis. In the monocytes, our earlier studies have established that US28 induces a G$\alpha$q→PLC-$\beta$→IP3 signaling pathway so we focused our studies on this pathway. In these experiments, since US28 inhibits basal cellular migration, we would predict that inhibiting key components of this signaling pathway would restore basal migration (i.e rescue the effects of US28). Pre-treatment of US28 expressing

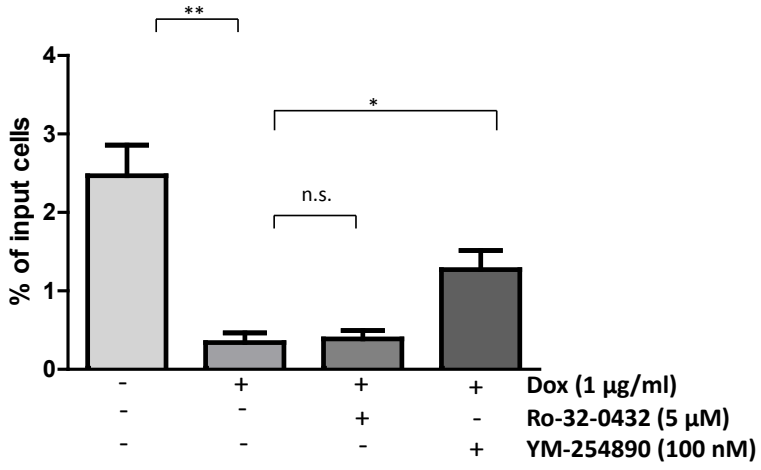



**Figure 23. US28 induced Gαq signaling is required for inhibition of THP-1 chemokinesis**
Transduced THP-1 cells were treated with 1 µg/ml of doxycycline or vehicle ($H_2O$) for 24 hours to induce US28 expression and cells were treated with Gαq inhibitor (YM-254890), PKC inhibitor (Ro-32-0432), or vehicle (DMSO). Transwell assays were performed for 2 hours. Numbers of input cells and cells migrating to the bottom chamber were determined by flow cytometry. The ratios between cells in the bottom chamber and input cells were converted into percent migration. The graph represented four independent experiments. Unpaired Student t test was used to analyze the data. **: $p<0.01$, *: $p<0.05$, n.s.: not statistically significant.

cells with the Ga $\alpha$ q inhibitor, YM-254890 led to a significant recovery in cell migration, indicating that US28 signaling through Gαq plays an important role in down-regulation of THP-1 migration (Figure 23).

Protein kinase C (PKC) is known to be a downstream kinase in the Gαq signaling pathway(225,226), and to determine whether US28 inhibits THP-1 through PKC, PKC inhibitor RO-32-0432 (214) was used to treat US28 expressing cells. RO-32-0432 treatment did not rescue the inhibitory effect of US28 on cell migration (Figure 23). Therefore, PKC is not involved in modulation of US28 induced inhibition of THP-1 migration.

**US28 promotes THP-1 monocyte adhesion to endothelial cells**

Monocytes, a cell type in which HCMV establishes latency, typically circulate throughout the blood system, and when stimulated by inflammatory signals, they extravasate across the endothelial lining into surrounding tissues where they differentiate into macrophages (80,227). In the case of HCMV infected monocytes, as monocyte→macrophage differentiation proceeds during and after adhesion to the endothelial cell layer (81,91,95), HCMV is thought to transition from a latent stage into a lytic one. Since US28 has a dramatic effect on monocyte migration in our THP-1

system, we hypothesized that US28 might also affect adhesion of the THP-1 cells to an endothelial monolayer. THP-1 cells induced to express US28 via DOX addition were added to endothelial cell monolayers and allowed to adhere for a time course between 5 and 60 minutes. We chose this time course based on published studies by other groups using THP-1 cells as a model for endothelial adhesion (228-230). At each time point, non-adhered THP-1 cells were removed from the endothelial monolayer by washing twice with culture media. Monolayers containing endothelial cells and adhered THP-1 cells were harvested and stained with the CD36, which is expressed on THP-1 cells but not on the underlying endothelial monolayer. US28 expressing cells showed a significant increase in endothelium adhesion at all time points tested (Figure 24).

Cells expressing either wildtype US28 or the DN US28 mutant exhibited a 7-10% increase in cell adhesion at 60 minutes, while cells expressing the US28 R129A mutant do not demonstrate any increase in cell adhesion (Figure 25). Based on the data with US28 mutants, these data again imply that US28 derived signaling may play an active role in facilitating cell adhesion to endothelial cells (Figure 25).

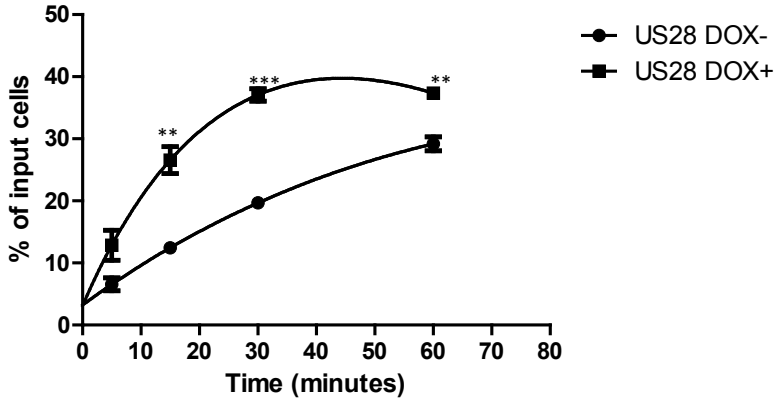



**Figure 24. US28 promotes monocyte-endothelial adhesion.**

Transduced THP-1 cells were treated with doxycycline (1µg/ml) or vehicle (water) for 24 hours to induce US28 expression. Cells were then co-cultured with and HECV cell endothelial monolayer for indicated times. Unattached cells were removed by washing with media. The number of attached cells and input cells were counted by flow cytometry. The data represent 3 independent experiments performed in duplicate. ** : $p<0.01$, *** : $p<0.001$

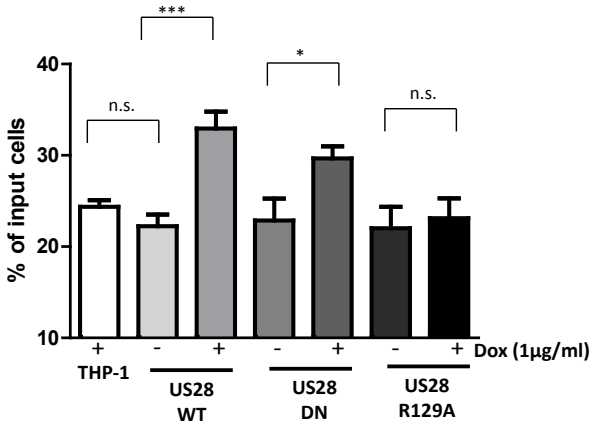



**Figure 25. US28 induced constitutive signaling regulates monocyte-endothelium adhesion.**

Transduced THP-1 cells were treated with 1 µg/ml of doxycycline or vehicle ($H_2O$) for 25 hours to induce US28 expression. $5x10^5$ THP-1 cells were co-cultured with HECV cells for one hour, and unattached cells were washed away with media. Numbers of input cells and cells attaching to HECV cells were determined by flow cytometry. The ratios between cells attaching to HECV cells and input cells were converted into a percentage. The graph represented eight independent experiments. Unpaired Student t test was used to analyze the data. ***: $p<0.0001$, *: $p<0.05$, n.s.: not statistically significant.

It is especially intriguing that the US28 DN mutant induced monocyte adhesion similarly to that of US28 WT. US28 has been shown to be able to bind to fractalkine(231), an adhesion molecule which can be expressed by endothelial cells(232,233). Therefore, it was possible that US28-mediated monocyte adhesion to endothelial cells would be occurring through a mechanism involving binding of US28 to fractalkine present on the endothelial cell surface. However, our results show that this is not the mechanism that US28 uses to promote monocyte-endothelial cell adhesion because US28 and DN US28 can promote monocyte adherence to endothelial cells while US28 R129A does not. Therefore, based on these data we conclude that ligand binding is not involved in the US28-mediated monocyte/endothelium adhesion observed in our studies. Rather, the data support the notion that US28 signaling is an important factor in this process.

To explore the nature of the constitutive US28-based signaling involved in regulation of monocyte-endothelial adhesion, we investigated the effects of the Gαq and PKC inhibitors that we used previously in signaling and chemotaxis assays. The Gαq specific inhibitor YM-254890 and PKC inhibitor Ro-32-0432 were both validated

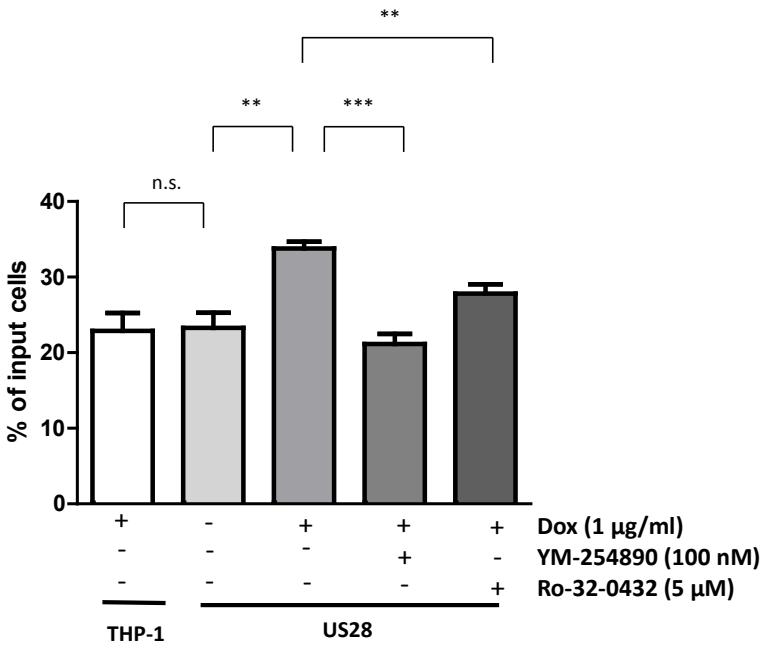



**Figure 26. US28 induced activation of Gαq and PKC promotes monocyte-endothelium adhesion.**

Transduced THP-1 cells were treated with 1 µg/ml of doxycycline or vehicle ($H_2O$) for 24 hours to induce US28 expression. Induced cells were then pretreated with inhibitors of Gαq (YM-254890, 100 nM), PKC (Ro-32-0432, 5 µM), or vehicle for an hour. $5x10^5$ THP-1 cells were co-cultured with HECV endothelial cells for one hour and unattached cells were washed away with media. Numbers of input cells and cells attaching to HECV cells were determined by flow cytometry. The ratios between cells attaching to HECV cells and input cells were converted into a percentage. The graph represents four independent experiments. Unpaired Student t tests were used to analyze the data. **: $p<0.01$, ***: $p<0.0001$, n.s.: not statistically significant.

prior to being used in these experiments (Figures 21 and 23). Cells were treated with DOX overnight to induce US28 expression and then treated with inhibitors for 1 hour before being overlaid onto the endothelial cells. Both inhibitors abolished the US28 induced increase in cell adhesion, showing that US28 induced Gαq dependent PKC activation is involved in regulation of monocyte-endothelium adhesion (Figure 26).

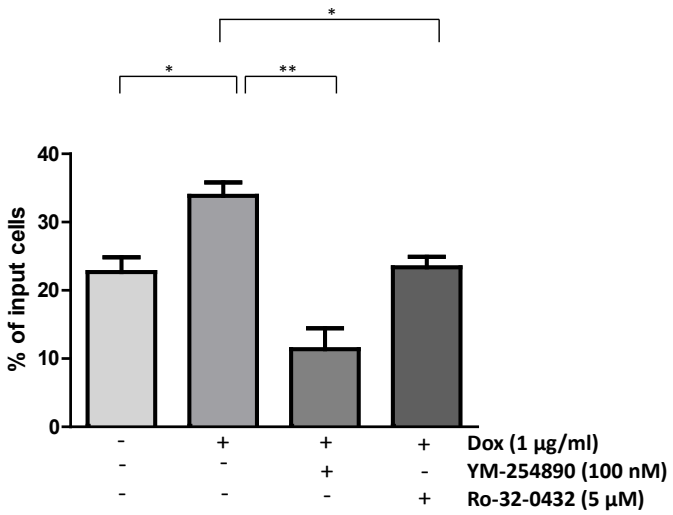



**Figure 27. US28 induced activation of Gαq and PKC promotes THP-1 adhesion to collagen.**

Transduced THP-1 cells were treated with 1 µg/ml of doxycycline or vehicle ($H_2O$) overnight to induce US28 expression. Cells were pretreated with inhibitors of Gαq (YM-254890, 100 nM), PKC (Ro-32-0432, 5 µM), or vehicle for one hour prior to being overlayed onto HECV endothelial cells. $5x10^5$ THP-1 cells were added to wells of a 24 well-plate that had been pre-coated with collagen. Cells were allowed to adhere for one hour, and unattached cells were washed away with media. Numbers of input cells and attached cells were determined by flow cytometry. The ratios between cells attaching to the plate and input cells were converted into percentage. The graph represents 3 independent experiments performed in duplicate. Unpaired Student T tests were used to analyze the data. * : $p<0.05$, **: $p<0.01$.

Although in the monocyte-endothelium adhesion assay, the media used for the assays does not contain inhibitors, it is possible that trace levels of the inhibitors could be carried over thereby influencing monocyte-endothelial adhesion by modulating the phenotype of the endothelial cell but not the monocyte. To address this issue we examined THP-1 monocyte adhesion to plastic culture dishes that were coated with collagen. Interestingly, US28 also promoted the ability of THP-1 monocytes to adhere to the collagen coated plastic (Figure 27). Therefore, we conclude that the increased adherence of THP-1 monocytes is due to a direct effect of US28 signaling on the phenotype of the THP-1 monocytes. Since our data indicated that pharmacological inhibition of G$\alpha$q and PKC signaling pathways blocked monocyte/endothelial adhesion (Figure 26) we predicted that inhibition of these pathways would similarly block direct monocyte adhesion to collagen. As hypothesized inhibition of G$\alpha$q (with YM-254890) and PKC (with Ro-32-0432) caused significant decreases in US28 induced collagen binding confirming that both Gαq and PKC are essential for US28 induced c ell adhesion phenotype (Figure 27).

In summary, we demonstrate that US28 signaling modulates both monocyte migration and monocyte-endothelial adhesion. Moreover, both of these processes involved classical G-protein signaling via the G$\alpha$q pathway.

**Discussion**

In this research, we demonstrate that US28 protein is expressed in latently infected THP-1 cells, and that ectopic expression of US28 in THP-1 cells leads to constitutive activation of Gαq signaling, which leads to the inhibition of cellular chemokinesis and induction of THP-1/endothelial cell adhesion. Our results provide novel insights into the function and signaling of US28 in a cell type that can serve as a reservoir for HCMV latency. Since monocyte migration and adhesion to endothelial cells are cellular properties important for viral dissemination (80), our results raise the possibility that US28 may play an essential role in HCMV dissemination. In murine cytomegalovirus (MCMV) studies, it has been shown MCMV M33-null virus has a defect in viral dissemination as it fails to grow in the salivary gland and a US28 rescued virus showed partially restoration in salivary gland growth (198), supporting the hypothesis that US28 can influence viral dissemination.

Our studies primarily use inducible US28 expressing THP-1 cells to explore the functional activities and signaling pathways induced by US28 expression because only 5-8% of monocytes or THP-1 cells express HCMV genes after infection (Chapter 2) and it is difficult to examine signaling and function when the uninfected cells outnumber the infected cells by such a large margin. In addition, as HCMV encodes over 200 gene products, many of which influence cell signaling and could thereby mask the effects of US28, it is important to first examine US28 function in isolation in

specific cell types and then to revisit interesting questions at a later point in the context of infection. Due to these limitations, we chose to use an inducible ectopic expression system to investigate the signal and function of US28 in THP-1 cells. Once the effects of US28 in monocytes and THP-1 cells is determined, pure infected cell populations could be established by flow cytometry or drug selection and used to confirm US28 function and signaling in the context of infection context. We are currently performing experiments in primary monocytes to examine US28 protein expression and examining whether US28 inhibits chemokinesis and promotes cell adhesion in primary cells are future directions for our studies.

We provide evidence that US28 can inhibit monocyte migration in a Gαq-dependent, but PKC-independent fashion. Further research is needed to explore the downstream signal molecules that regulate the US28 induced down-regulation of monocyte migration. One interesting potential avenue could be whether or not US28 alters basal cytoskeleton function in these cells. G$\alpha$q is linked to Rho signaling via p63Rho-GEF (234) and would be interesting to explore a link between constitutively active G$\alpha$q and the cytoskeleton (235). The concept of “constitutive” signaling remains an enigma regarding downstream processing and it is interesting to speculate that chronic activation of p63Rho-GEF could actually have a deleterious effect on cytoskeletal regulation.

We also provide evidence that US28 promotes monocyte/endothelial adhesion via the activation of a Gαq→PLC-β→PKC pathway. The mechanism of US28 promoted adhesion is somewhat surprising given that US28 can bind cell surface ligands such as fractalkine(231), but it is clear from our mutant studies that the amino-terminus of US28 is not required for this activity and that pharmacological inhibition of monocyte signaling pathways abolishes this activity. The target(s) downstream of PKC in this process have not yet been identified yet. One possible candidate PKC target is α4β1 integrin. Previous studies have demonstrated that PLC-β activation leads to an inside-out signal for α4β1 integrin activation (236) which causes increased adhesion to VCAM-1 and fibronectin found on endothelial cells (228).

HCMV infection has frequently been reported as a potential co-factor in the promotion of atherosclerosis (83,162,237). Several groups have speculated that US28 could impact the progression of atherosclerosis via the stimulation of smooth muscle cell migration (79,238,239). However, many studies have implied that monocyte-endothelium adhesion is a crucial factor for atherosclerosis (240-242) and our results suggest that US28 could have an additional function in the development or promotion of atherosclerosis (184) by promoting monocyte adhesion to the endothelium adjacent to the developing plaque. Future research and the development of appropriate animal models will be needed to verify whether US28 indeed can affect

atherosclerosis.

The ability of US28 to activate Protein kinase C may have broad implications beyond the scope of the current study. PKC is known to affect the differentiation of myeloid cells(243,244), a process clearly central to the HCMV latent→lytic switch(19). We have not observed any significant effect of US28 on myeloid maturation and differentiation in the THP-1 model (data not shown). However, it is possible that US28 signaling through PKC may have profound effects on the differentiation of myeloid progenitor cells and influence progression of HCMV into the lytic phase. Cord-blood derived CD34+ hematopoietic progenitors (HPCs) (72) and the Kasumi-3 cell line (120) have both been appropriate for HCMV latency studies, and it would be intriguing to determine whether US28 signaling through PKC can influence HPC and/or Kasumi-3 differentiation.

In summary, in these studies we demonstrate that US28 protein is expressed in non-permissively infected myeloid cells and has the potential to regulate monocyte migration and adhesion. Therefore, we speculate that US28 not only has important functions in the permissive phase but also may play a crucial role in viral latency in myeloid cells and progenitors, establishing US28 as an important target for novel therapeutics to combat HCMV pathogenesis and disease.

# Chapter V.

# Discussion and future directions

In Chapter II of this thesis, we demonstrate that vitamin D (1,25-dihydroxyvitamin D3) treatment of THP-1 cells or primary monocytes can lead to maturation/differentiation of monocytes and support permissive infection of HCMV. One of the future directions that we want to investigate is to assess the influence of vitamin D treatment on HCMV replication in hematopoietic progenitor cells. Vitamin D treatment has been reported to promote monocyte-macrophage lineage commitment in hematopoietic progenitor cells (150). Therefore, we hypothesize that vitamin D treated hematopoietic progenitor cell could have an increased percentage of cells that are permissive for HCMV lytic replication due to an increased number of hematopoietic progenitor cells committed to the monocyte-macrophage lineage.

In addition, the effect of vitamin D on HCMV reactivation is another area of interest that has not been explored. To investigate this point, hematopoietic progenitor cells will be infected with HCMV using conditions that favor establishment of latency. Once the cells have established a latent infection, they will be treated with vitamin D for two weeks. Based on published results, two weeks has been established as an appropriate time point to promote an increased commitment to the monocyte-macrophage lineage (150). Since it is known that HCMV reactivates from latency when monocyte differentiates into macrophage or mature dendritic cell (245), vitamin D may increase the likelihood that HCMV reactivation will occur by

augmenting the number of cells committed to the monocyte-macrophage lineage.

Since Vitamin D has such a profound effect on HCMV replication in human monocytes, we chose to use the murine cytomegalovirus (MCMV) system to ask whether Vitamin D and the Vitamin D receptor (VDR) would similarly affect MCMV replication in an in vivo model. Unfortunately, our preliminary data using wildtype and $VDR^{-/-}$ mice did not reveal a significant difference in MCMV titers in the spleen at day 4 post-infection. However viral replication parameters during earlier stages of infection (days 1-3) during which innate immunity plays a more central role in controlling CMV infection (164,165) has not been investigated. Given that the action of monocytes and other innate immune cells towards CMV infection can happen within 2 days of the initial infection, it would be intriguing to monitor immune system responsiveness and viral titers in multiple organs at the very earliest stages of infection. Another explanation for the observation that no viral replication differences were identified in VDR knockout mice could be that while vitamin D has been shown to regulate innate and adaptive immune responses in mice (246), some of the classical VDR target genes in human are not regulated by VDR in mice. For example, the cathelicidins, a family of anti-microbial peptides, are known to be regulated by VDR in human, but not in mouse (168). Therefore, the further refinement of the molecular mechanisms used by vitamin D to facilitate HCMV replication in monocytes is essential. It is entirely

possible that VDR upregulates a set of genes in human monocytes would also facilitate CMV replication in the murine system if those same genes were upregulated by VDR in mice. RNA-Seq type analysis of Vitamin D/VDR induced gene expression in the human and murine systems is likely to reveal important insight into not only the mechanism(s) of VDR action but also into the molecular details underlying the onset of lytic CMV replication in myeloid cells.

While, the molecular mechanism(s) that vitamin D employs to facilitate HMCV permissive infection in monocytes remains unknown, previous studies indicated that the Bcl-6 transcription factor is upregulated in THP-1 cells after vitamin D treatment(247,248), and it is known that Bcl-6 can have an opposing effect on several of the NF-κB regulated inflammatory genes(249,250). The role of NF-κB in HCMV replication is still controversial. Although some studies indicate that NF-κB can promote HCMV replication (251-253), others show that activation of NF-κB can inhibit HCMV replication (254,255). An explanation for these disparate results may be the different methodologies used in each study, or differences in downstream gene expression in the exact cell type used in each experiment setting. Therefore, we hypothesize that Bcl-6 could play a role in modulating the expression NF-κB dependent inflammatory genes that are also triggered upon HCMV infection. Thus, Bcl-6 upregulation by Vitamin D could create an environment that more strongly

supports HCMV lytic infection in THP-1 cells and primary monocytes. Moreover, Bcl-6 has been known to polarize macrophages into the M2 subtype which participates in dampening immune responsiveness and tissue repair.(256,257) An unrelated study demonstrated that the percentage of IE positive cells in TB40E infected M2 macrophages is about 5 fold higher than that in M1 macrophages (258). Taken together, these findings suggest that Bcl-6 activity could trigger a select set of target genes thus providing a potential molecular mechanism underlying the onset of HCMV lytic replication in myeloid cells.

In Chapter III, we demonstrate that YM-254890 compound exhibits a potent inhibitory effect that can block cytomegalovirus vGPCR induced Gαq dependent signaling pathways. Due to fact that cytomegalovirus vGPCRs expressed on different cell types may trigger distinct signaling activities or cause tissue specific responses, this inhibitor (and related synthetic compounds like WU-0747) may prove to be valuable tools that we could use to explore the signaling pathways and cellular responses affecting viral replication in various cell types and *in vivo*. The information gleaned from such studies will help us to correlate cellular responses induced by vGPCR signaling with viral replication in those cells and could potentially be exploited as novel anti-virals.

Finally, in Chapter IV, we demonstrated that US28 protein is expressed in HCMV

infected monocytes where it can trigger a constitutive Gαq dependent signal. This US28 induced constitutive signal promotes monocyte adhesion and monocyte-endothelial cell adhesion but inhibits THP-1 chemokinesis. These results suggest that US28 may play a role in viral dissemination through modulation of monocyte adhesion and migration.

A potential role for US28 in viral dissemination draws parallels with studies involving the MCMV encoded vGPCR M33. Studies with MCMV and M33 have shown that deletion of M33 (which like US28 is also a Gαq coupled GPCR) causes a defect in viral dissemination to salivary glands(196). Interestingly, insertion of the HCMV US28 gene into the M33 null virus genome partially rescues the viral dissemination exhibited by loss of M33 (198) suggesting that US28 may similarly participate in viral dissemination in that model system.

Since US28 transcripts can also be detected in hematopoietic and myeloid progenitor (27,72), we are keenly interested in pursuing studies aimed at discerning the function of US28 in these cell types. Kasumi-3 is a myeloid progenitor cell line that has recently been shown to be a useful model to study both latent and permissive infection of HCMV (120). We will use this cell line to study the function of US28 in myeloid progenitor cells. Based on our studies with Vitamin D in THP-1 cells it is quite evident that tipping the balance and driving monocytes into mature monocytes is

sufficient to trigger lytic replication. From our signaling studies in THP-1 cell, we can demonstrate that US28 signaling can activate protein kinase C, and based on previous studies it is clear that PKC is centrally involved in regulation of myeloid cell differentiation (243,244). In THP-1 cells, we did not identify significant effect of US28 on the expression of cell differentiation markers, but whether US28 could influence the differentiation of myeloid progenitor cells is an intriguing direction for future studies. Kasumi-3 cells will be used to investigate the impact of US28 on cell differentiation. A similarly interesting direction for the future study is to examine whether US28 can affect latency or reactivation. Previous studies have shown that HCMV can establish latency in Kasumi-3 cells, and unlike THP-1 cells, HCMV can be reactivated from latency in Kasumi-3 cells (120). Inducible US28 expressing Kasumi-3 cell line will be utilized to study the effect of US28 on latency or reactivation.

In summary, the future directions discussed briefly in this chapter will provide a deeper understanding of the molecular mechanisms underlying the host-pathogen interactions, which influence HCMV replication and dissemination in myeloid cells. In doing so, we could not only begin to unravel the mechanisms underpinning Vitamin D's effect on HCV replication, but also begin to link viral (i.e. US28) and cellular factors (i.e. VDR, Bcl-6, etc) that could be potential targets for novel HCMV therapeutics.

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