ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
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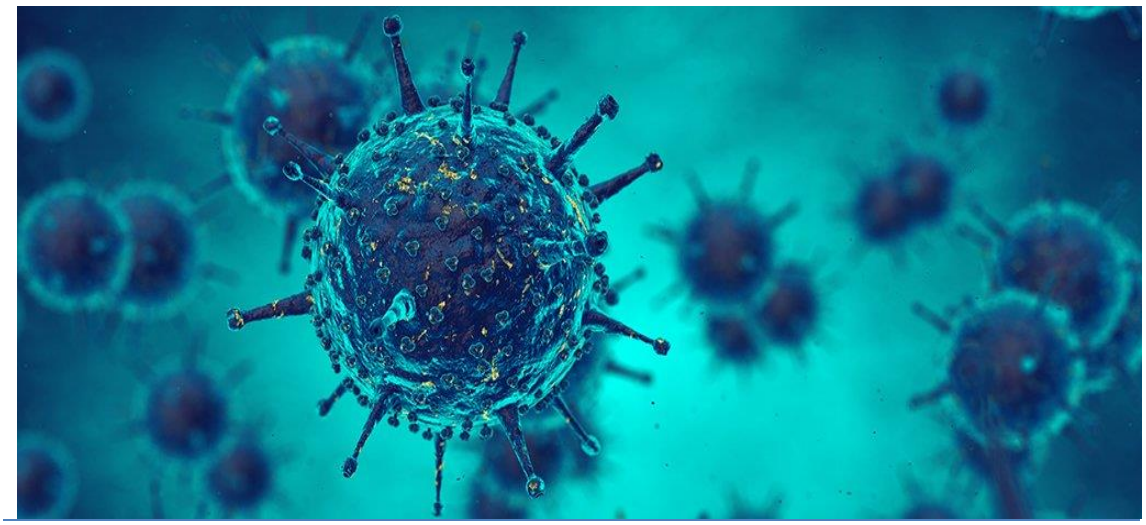
ΕΡΓΑΣΤΗΡΙΟ ΚΛΙΝΙΚΗΣ ΙΟΛΟΓΙΑΣ

Ο ρόλος των πρωτεϊνών Rho GTPases κατά τη διάρκεια της μόλυνσης από τον ανθρώπινο κυτταρομεγαλοϊό.

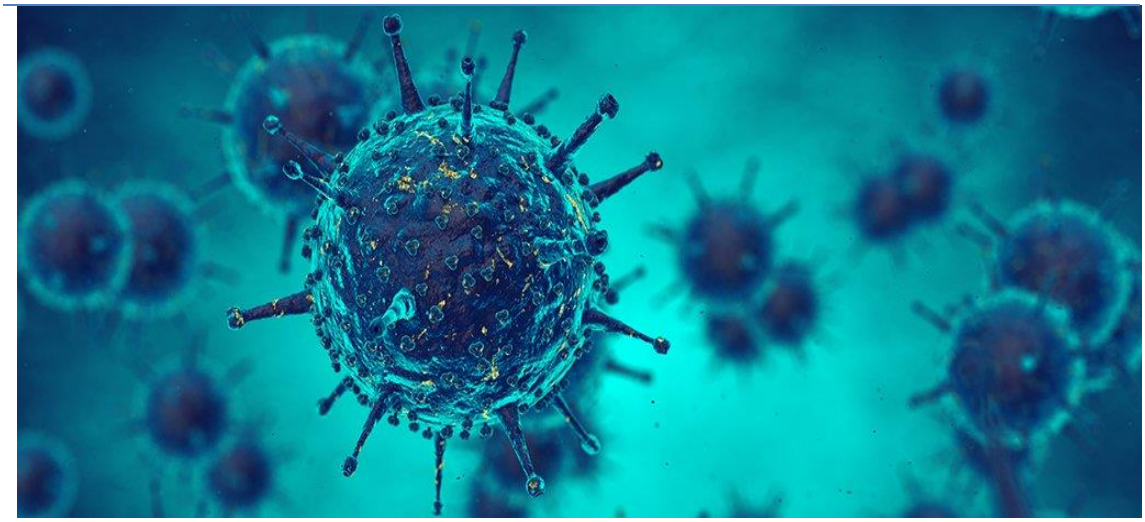


ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ
ΜΕΛΠΟΜΕΝΗ ΤΣΕΛΙΟΥ

ΗΡΑΚΛΕΙΟ 2017

UNIVERSITY OF CRETE
MEDICAL SCHOOL
LABORATORY OF CLINICAL VIROLGY

The role of Rho family GTPases in Human Cytomegalovirus infection (Human Cytomegalovirus, HCMV).



DOCTORAL THESIS

MELPOMENI TSELIOU

HERAKLION 2017

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RESEARCH

Research experience

1. 04/2013-today: I performed my PhD at the Laboratory of Clinical Virology, University of Crete, Medical School, under the supervision of the professor of Clinical Virology George Sourvinos.

   Projects:

   a. Study of the role of small Rho GTPases in Human Cytomegalovirus infection and pathogenesis (HCMV).

   b. Study of the antiviral activity of various plants extracts during lytic infection by HCMV. Plant extracts tested: dandelion root and leaf extract and a herbal extract of a blind trial.

   c. Study of the antiviral activity of various plant extracts during infection by different influenza virus strains. Plant extracts tested: coridothymus capitatus, salvia fruticosa and origanum dictamnus.

2. 02/2012-11/2012: I performed my master thesis at the Laboratory of Pharmacology, University of Crete, Medical School, under the supervision of the professor of Pharmacology Kiriaki Thermos.

   Project: Study of the involvement of NO and cGMP in the neuroprotective effects of somatostatin in the in vivo model of AMPA-induced excitotoxicity in rat retina.

3. 11/2011-01/2012: Lab rotation at the Laboratory of Pharmacology, Unifersity of Crete, Medical School, under the supervision of the assistant professor of Pharmacology Ioannis Charalampopoulos.

   Project: Study of the role of neurosteroid dehydroepiandrosterone (DHEA) in neuronal apoptosis.

4. 08/2011-10/2011: Lab rotation at the Laboratory of Pharmacology, University of Crete, Medical School, under the supervision of the professor of Pharmacology Kiriaki Thermos.

   Project: Study of the neuroprotective effects of the venzoxazines in the in vivo model of AMPA-induced excitotoxicity in rat retina.

5. 05/2011-07/2011: Lab rotation at the Laboratory of Molecular Biology/Developmental Neurobiology, University of Crete, Medical School, under the supervision of the professor of Molecular Biology Domna Karagogeos.

   Project: Study of the role of TAG-1 in the organization of myelin axons, during demyelination and remyelination.

6. 09/2009-02/2010: Undergraduate thesis: "Study of the effects of cigarette smoke exposure and its cessation on body weight, food intake and circulating leptin, and ghrelin levels in the rat'', Democritus University of Thrace, Medical School, Biochemistry Laboratory, supervisor: assistant professor of Biochemistry Konstantinos Anagnostopoulos.

7. 03/2009-08/2009: undergraduate training at the Laboratory of Experimental Surgery and Surgical Research, University General Hospital of Alexandroupolis, Laboratory of Experimental Surgery, supervisor: associate professor of Experimental Surgery Petros Ypsilantis.

Laboratory skills

Cell culture, virus propagation and titration, adenoviral/retroviral vectors, silencing techniques, immunofluorescence, microscopy (live-imaging, fluorescence, confocal), western blots, cytoplasmic and nuclear extract fractionation, flow cytometry, RT-PCR, qPCR, transfection, laboratory mice and rats handling etc.

Publications

1. **Tseliou M**., Al-Qahtani A., Alarifi S., Stournaras C., Sourvinos G. **The role of RhoA, RhoB and RhoC GTPases in cell morphology, proliferation and migration in human cytomegalovirus (HCMV) infected glioblastoma cells.** Cell Physiol Biochem2016;38(1):94-109. doi: 10.1159/000438612. Epub 2016 Jan 8.

2. Al-Qahtani AA., Lyroni K., Aznaourova M., **Tseliou M**., Al-Anazi MR., Al-Ahdala M., Alkahtani S., Sourvinos G., Tsatsanis C. **Middle east respiratory syndrome corona virus spike glycoprotein suppresses macrophage responses via DPP4-mediated induction of IRAK-M and PPARγ.** Oncotarget. 2017 Jan 19. doi: 10.18632/oncotarget.14754.

Conferences

1. **Tseliou M.**, Al-Qahtani AA., Aznaourova M., Al-Anazi MR., Al-Ahdal MN., Alkahtani S., Tsatsanis C., Sourvinos G. Middle east respiratory syndrome corona virus spike glycoprotein suppresses macrophage responses via DPP4-mediated induction of IRAK-M and PPARγ, 13th

Greek Conference of Clinical Chemistry, 29-31 October 2015, Medical School, University of Crete, Heraklion, oral presentation.

2. **Melpomeni Tseliou**, Saud A. Alarifi, Saad H. Alkahtani, Ahmed Al-Qahtani, Christos Stournaras4, George Sourvinos. Differential role of Rho small GTPases in proliferation and migration in human cytomegalovirus (HCMV) infected glioblastoma cells. European Society of Clinical Virology, 9-12 September 2015, Edinburgh, Scotland UK Journal of Clinical Virology 70 (2015) S1-S126, poster.

3. **Melpomeni Tseliou**, Saud A. Alarifi, Saad H. Alkahtani, Ahmed Al-Qahtani, Nektaria Goulidaki, George Sourvinos. The effect of Rho small GTPases signaling in human cytomegalovirus (HCMV) infected fibroblasts and glioblastoma cells. Society for General Microbiology Annual Conference 2015, 30 March-2April 2015, Birmingham, The Intrenational Convention Centre, poster.

4. **Melpomeni Tseliou**, Saud A. Alarifi, Saad H. Alkahtani, Ahmed Al-Qahtani, Nektaria Goulidaki, George Sourvinos. The effect of Rho small GTPases signaling in human cytomegalovirus (HCMV) infected glioblastoma cells. 10th Congress of Virology, War Museum of Athens, 27-28 February, 2015, Athens Greece, poster.

5. **Melpomeni Tseliou**, Saud A. Alarifi, Saad H. Alkahtani, Ahmed Al-Qahtani, Nektaria Goulidaki, George Sourvinos. The effect of Rho small GTPases signaling in cytoskeleton and motility in human cytomegalovirus (HCMV) infected fibroblasts and glioblastoma cells. The 19th Congress of Advances in Oncology and 17th International Symposium on Molecular Medicine, 9-11 October, 2014, Metropolitan Hotel, Athens, Greece, International Journal of Molecular Medicine 34: Supplement, 2014, poster.

6. P. Ypsilantis, M. Politou, K. Anagnostopoulos, Ch. Tsigalou, **M. Tseliou**, E. Papanastasi, K. Simopoulos. The effects of cigarette smoke exposure and its cessation on body weight, food intake and circulating leptin, and ghrelin levels in the rat. 38th Conference of Endocrinology, 8-10 April, 2011, Thessaloniki, Greece, poster.

Grants / Awards

Scholarship and travel grant for attendance of the Intensive Programme ''PET Imaging'', March-April 2011, from the Institut National Des Sciences et Techniques Nucleaires, Saclay, France.

Memberships

1. European Society for Clinical Virology
2. Hellenic Society for Biochemistry and Molecular Biology

Work experience

Private tutor of biology preparing high school students for the Greek national examinations that grant access to universities.

Education

1. 04/2013- today: PhD student, University of Crete, Medical school, PhD thesis: "Study of the Role of Rho family GTPases in Human Cytomegalovirus infection and pathogenesis (HCMV)''. Laboratory of Clinical Virology, supervisor: professor of Clinical Virology George Sourvinos.
2. 09/2010 – 11/2012: Graduate studies, University of Crete, Medical school, graduate program "Neurosciences". Graduate thesis: ''The involvement of NO and cGMP in the neuroprotective effects of somatostatin in the in vivo model of AMPA-induced excitotoxicity in rat retina''.
3. 09/2005- 03/2010: Undergraduate studies, Democritus University of Thrace, Department of Molecular Biology and Genetics.
4. 2002-2005: Student, Senior high school of Karpenisi, Greece (orientation: exact sciences).
5. 1999-2002: Student, high school of Karpenisi, Greece.

Languages

1. Greek, native speaker
2. English : fluent
   Certificate of Competency in English, University of Michigan
3. French: fluent
   1er Diplome d' Etudes en Langue Francaise

IT skills

1. International Diploma in IT Skills Standard, University of Cambridge
2. Programming language C++
3. Competent user of Microsoft office, GraphPad and Photoshop

Ευχαριστίες

Η παρούσα διδακτορική διατριβή εκπονήθηκε στο εργαστήριο Κλινικής Ιολογίας του Πανεπιστημίου Κρήτης. Για εμένα αποτέλεσε μία τεράστια εμπειρία, με έντονες στιγμές, με απογοητεύσεις και χαρές, γεμάτη ενδιαφέροντες ανθρώπους και με βοήθησε να ωριμάσω τόσο σε επιστημονικό όσο και σε προσωπικό επίπεδο. Τελειώνοντας λοιπόν τη διδακτορική μου διατριβή θα ήθελα να ευχαριστήσω όλους τους ανθρώπους που συνέβαλαν σε αυτή είτε άμεσα είτε έμμεσα.

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Φυσικά δε θα μπορούσα να μην ευχαριστήσω όλα τα μέλη του εργαστηρίου Κλινικής Ιολογίας, νέα και παλιά για την ανεκτίμητη βοήθειά τους και το όμορφο κλίμα συνεργασίας στο εργαστήριο. Το μεγαλύτερο ευχαριστώ όμως το χρωστάω στην Εύη Βλαχάβα, που πέρα από την άψογη συνεργασία μας αποκτήσαμε και μια πολύ δυνατή φιλία. Ευάκι σ' ευχαριστώ πολυ για όλα!

Πολλά ευχαριστώ οφείλω και στους φίλους μου Στέλλα, Κέλλυ, Αντιγόνη, Χρύσα, Άννα, Ελίζα, Χρυσαλίντα και Χαρά. Ένα μεγάλο ευχαριστώ επίσης και στο Μίμη!

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Στους γονείς μου...

Contents

Abstract

Human cytomegalovirus (HCMV) is one of the eight human herpesviruses with worldwide distribution and a high clinical importance. It is a beta herpes virus with double stranded DNA and establishes a lifelong latent infection after a primary infection. Rho GTPases are crucial regulators of the actin cytoskeleton and play a role in controlling membrane trafficking and cell signaling. The actin cytoskeleton of the host cell and actin regulating Rho GTPase signaling pathways are involved in several of the interactions of human cells with viral components. This has provided scientific data so as to investigate further on the Human Cytomegalovirus (HCMV). So far, there is short evidence on the role of Rho GTPases during HCMV infection.

In the present thesis, we studied the mechanism that controls infection by HCMV investigating two directions; the effect of RhoA and the role of RhoA depletion during the course of infection and the effect of silencing RhoA, RhoB or RhoC in HCMV infected glioblastoma cells.

The first part of this thesis focused in the analysis of the effect of RhoA silencing during the various early and late stages of HCMV infection to establish whether RhoA and RhoA downstream effectors are directly involved in promoting productive HCMV infection. Therefore it was of high priority to investigate the effect of RhoA and the role of RhoA knockdown at the early stages of infection that occur immediately after viral uptake into the host cell as well at the later stages during viral egress. Collectively, it was demonstrated that RhoA knockdown provides benefit to early and late stages of HCMV infection by the inhibition of the RhoA and ROCK1 pathway.

At the second part of the study, we analyzed modifications in the organization of the actin cytoskeleton that are regulated by RhoA, RhoB and RhoC GTPases and affect cell motility and migration in HCMV infected cancer cells in order to explore properties of HCMV and its capability to modulate the tumor microenvironment. Taken together these data, we suggest a possible direct or indirect involvement of Rho small GTPases during HCMV infection with potential role in cell morphology, proliferation and migration of human glioblastoma cells.

Περίληψη

Ο ανθρώπινος κυτταρομεγαλοϊός (HCMV) είναι ένας από τους οχτώ ανθρώπινους ερπητοϊούς με ευρεία κατανομή ανά τον κόσμο και μεγάλη κλινική σημασία. Είναι ένας β-ερπητοϊός με διπή έλικα DNA που επιτυγχάνει μακροχρόνια λανθάνουσα φάση μετά από την πρωτογενή μόλυνση. Οι Rho GTPάσες είναι σημαντικοί ρυθμιστές της ακτίνης του κυτταροσκελετού και παίζουν σημαντικό ρόλο στη ρύθμιση της μεμβρανικής μεταφοράς και της κυυταρικής σηματοδότησης. Η ακτίνη του κυτταροσκελετού του κυττάρου ξενιστή καθώς επίσης και τα σηματοδοτικά μονοπάτια των Rho GTPασών που αυτή ρυθμίζει, εμπλέκονται στις αλληλεπιδράσεις των κυττάρων του ανθρώπου με ιικά στοιχεία. Αυτά τα επιστημονικά δεδομένα μας παρείχαν το έναυσμα για να μελετήσουμε αυτές τις αλληλεπιδράσεις στο πλαίσιο μόλυνς ης από τον HCMV. Παρ' όλα αυτά, υπάρχουν περιορισμένες μελέτες όσον αφορά το ρόλο των Rho GTPασών στη διάρκεια μόλυνσης από τον HCMV.

Στα πλαίσια της παρούσας διδακτορικής διατριβής διερευνήθηκαν οι μηχανισμοί που διέπουν τη μόλυνση από τον ανθρώπινο κυτταρομεγαλοϊό (HCMV). Συγκεκριμένα, διερευνήθηκε η δράση της RhoA GTPάσης και ο ρόλος της αποσιώπησής της στην έκβαση της μόλυνσης από τον HCMV. Επιπλέον, διερευνήθηκε η δράση των RhoA, RhoB και RhoC GTPασών και ο ρόλος της αποσιώπησής τους σε μολυσμένα από τον HCMV καρκινικά κύτταρα.

Στο πρώτο σκέλος της διατριβής διερευνήθηκαν τα φαινόμενα που προκαλεί η αποσιώπηση της RhoA GTPάσης στα πρώιμα και όψιμα στάδια της μόλυνσης από τον HCMV, με σκοπό να προσδιοριστεί ο ρόλος της RhoA GTPάσης καθώς επίσης και

των ρυθμιστικών της παραγόντων στην αποτελεσματική έκβαση της μόλυνσης από τον ανθρώπινο κυτταρομεγαλοϊό. Για το σκοπό αυτό μελετήσαμε το ρόλο της συγκεκριμένης GTPάσης στα πρώιμα στάδια της μόλυνσης που αρχίζουν με την πρόσληψη του ιού από το κύτταρο ξενιστή, καθώς επίσης και το ρόλο της RhoA GTPάσης στα όψιμα στάδια της μόλυνσης κατά τη διάρκεια των οποίων ο ιός εξέρχεται από το κύτταρο. Συνολικά, η αποσιώπηση της RhoA GTPάσης επιταχύνει τα διάφορα στάδια της μόλυνσης από τον HCMV μέσω της καταστολής του RhoA και ROCK1 σηματοδοτικού μονοπατιού.

Ως προς το δεύτερο σκέλος της εργασίας, διερευνήθηκαν οι αλλαγές στην οργάνωση της ακτίνης του κυτταροσκελετού που προκαλούνται από τις RhoA, RhoB και RhoC GTPάσες και ρυθμίζουν την κυτταροκίνηση και τις μεταναστευτικές ιδιότητες μολυσμένων από τον HCMV καρκινικών κυττάρων. Με αυτό το τρόπο μπορέσαμε να καθορίσουμε τις ογκοτρόπες δράσεις του HCMV καθώς επίσης και τη ικανότητά του να ρυθμίζει το μικροπεριβάλλον του καρκίνου. Συλλεγοντας όλα τα δεδομένα αυτού του σκέλους της εργασίας, προτείναμε μία άμεση ή έμμεση επίδραση των Rho GTPασών στη μορφολογία, τον πολλαπλασιασμό και τη μεταναστευτική ικανότητα μολυσμένων από τον HCMV κυττάρων γλοιοβλαστώματος.

1. Introduction

Herpesviruses

Classification of Herpesviruses

The family Herpesviridae consists of three subfamilies: Alphaherpesvirinae (containing the Simplexvirus, Varicellovirus, Mardivirus and Iltovirus genera), Betaherpesvirinae (containing the Cytomegalovirus, Muromegalovirus and Roseolovirus genera) and Gammaherpesvirinae (containing the Lymphocryptovirus and Rhadinovirus genera). The first attempt to classify herpesviruses was undertaken in 1971 by the International Committee on Taxonomy of Viruses (ICTV) mainly on the basis of structure. In general, the classification of herpesviruses incorporates morphological, genomic and serological criteria. Though morphological criteria have a prominent role in the classification of herpesviruses, recent advances in sequencing methods have dominated herpesvirus taxonomy (Figure 1.1) [1].

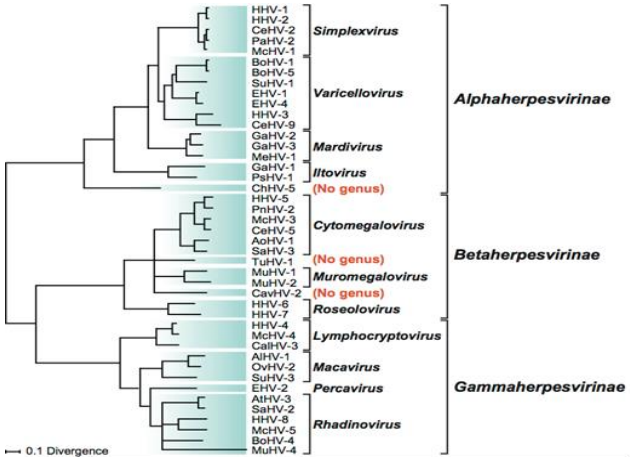


Figure 1.1: Phylogenetic relationships within the family Herpesviridae. (Adapted from ICTV 9th Report Taxonomy (2011) [2]).

In order for a virus to be classified as a member of the Herpesviridae family it needs to satisfy some fundamental morphological criteria. The virion should be spherical, consist of an icosahedral capsid, a tegument and an envelope and the genome inside the capsid should be linear double stranded DNA [3].

Genomic criteria have been used and refer mainly to the genome structure but this is not particularly useful since similar structures are common among the family. However, sequencing data, both from nucleotide and amino acid sequences, are of great significance to herpesvirus taxonomy [3].

Serological criteria are more useful, but only on the basis of characterizing closely related viruses, while biological criteria mainly refer to the host. Herpesviruses are particularly adapted to their host and can cause severe disease mainly to immunocompromised individuals. Individual herpesvirus species usually infect a specific host though cross-species transmission can occasionally occur. They are transmitted via aerosols or via contact with infected mucosal surfaces and they have evolved intricate mechanisms to cause lifelong latent infections.

Alphaherpesvirinae are characterized by a variable host range, a short life cycle, a rapid spread in culture conditions, an intense cytopathic effect and an ability to establish latent infections mainly in sensory ganglia. Among others the Alphaherpesvirinae contain the human affecting genera Simplexvirus (HSV-1 or HHV1 and HSV-1 or HHV2) and Varicellovirus (VZV or HHV3). Betaherpesvirinae, in contrast to alphaherpesviruses are more restricted in their host range, they progress slowly in culture conditions and they have a long life cycle. They also cause a

distinctive enlargement of their host cells called cytomegalia. Betaherpesviruses establish latent infections as well, but in contrast to alphaherpesviruses they do so in secretory glands, lymphoreticular cells, kidneys and other tissues. Betaherpesvirinae genera that contain human herpesviruses are the Cytomegalovirus genus (HCMV or HHV5) and the Roseolovirus genus (HHV6 and HHV7).

Gammaherpesvirinae limit their host range to a family of organisms. They are lymphotropic viruses and they can all replicate in lymphoblastoid cells in vitro. Some can additionally cause lytic infection to epithelial cells and fibroblasts. However, gammaherpesviruses are usually specific for B or T lymphocytes. There are two genera containing human herpesviruses in this subfamily, the Lymphocryptovirus genus (EBV or HHV4) and the Rhadinovirus genus (Kaposi's sarcoma-associated herpesvirus or HHV8) [4].

Within the Herpesviridae family, the alpha-, beta- and gammaherpesviruses have arisen from a common ancestor. Although there is apparently little genetic similarity between the three subfamilies, the capsid structures are highly conserved and it is well accepted that herpesviruses evolved in parallel with their hosts [5].

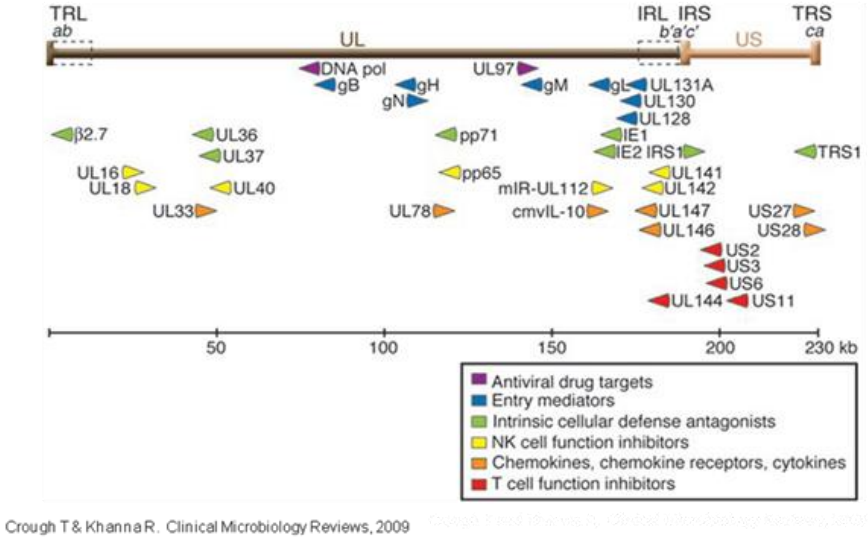
Human Cytomegalovirus, HHV5

General Information

Viruses have co-evolved with their hosts and have acquired strategies to corrupt cellular pathways in order to acquire viral replication and expansion. Human cytomegalovirus (HCMV) is one of the eight human herpesviruses with worldwide distribution and a high clinical importance. It can cause significant complications during pregnancy and in clinical situations associated with inefficient immunocompetence, such as organ or bone marrow transplantation, cancer and AIDS. HCMV has evolved a variety of interactions with host cells to create an optimal niche for viral replication, persistence and spread [6].

HCMV Genome

Human cytomegalovirus (HCMV) which is a beta herpes virus with double-stranded DNA, establishes a lifelong latent infection after a primary infection [7]. HCMV has the largest genome among all herpes viruses, approximately 230 kbp that is divided into a unique large (UL) and unique short (US) region (Figure 1.2).



Crough T & Khanna R. Clinical Microbiology Reviews, 2009

Figure 1.2: HCMV genome.

HCMV encodes over 200 open reading frames, but the exact number is depending on the strain [8]. As it mentioned above, it is known for its opportunistic infection in immunocompromised individuals such as in AIDS patients, organ and stem cell transplant patients and it is the main etiological agent responsible for congenital diseases in newborn babies [9]. Replication of HCMV initiates in the nucleus of host cells and is regulated by the expression of the immediate early (IE) genes which are regulatory genes controlling early and late genes expression in infected cells [10]. HCMV IE genes 1 and 2 (IE1 and IE2) are activated primarily in infected cells. IE1 and IE2 proteins regulate transcription of viral and cellular genes during HCMV infection and have been implied in the pathogenesis of many diseases [11].

Virus infectivity

Human cytomegalovirus (HCMV) infects 40% to nearly 100% of the adult population worldwide [12] and can also be acquired in the neonatal period via breast milk. The transmission rate from mothers to infants has a large scale variance (6%–60%) (Figure 1.3) [13]. Similar to other herpesviruses, HCMV establishes a persistent infection, remaining silent in the host and undergoing productive reactivation cycles for its efficient transmission. HCMV infects and replicates in a wide variety of cells, including epithelial cells of gland and mucosal tissue, smooth muscle cells, fibroblasts, macrophages, dendritic cells, hepatocytes and vascular endothelial cells [14]. This broad cell tropism facilitates systemic spread in the human body. In addition, HCMV undergoes latency in myeloid cells of the bone marrow, which probably leads to a life-long infection with sporadic reactivation [15]. HCMV infection is generally asymptomatic in healthy individuals. However, in

immunocompromised individuals HCMV poses a life-threatening risk [16]. The association of this virus with human diseases is highlighted by the decisions of the Institute of Medicine and the National Vaccine Advisory Board to assign HCMV as high priority for vaccine development [17].

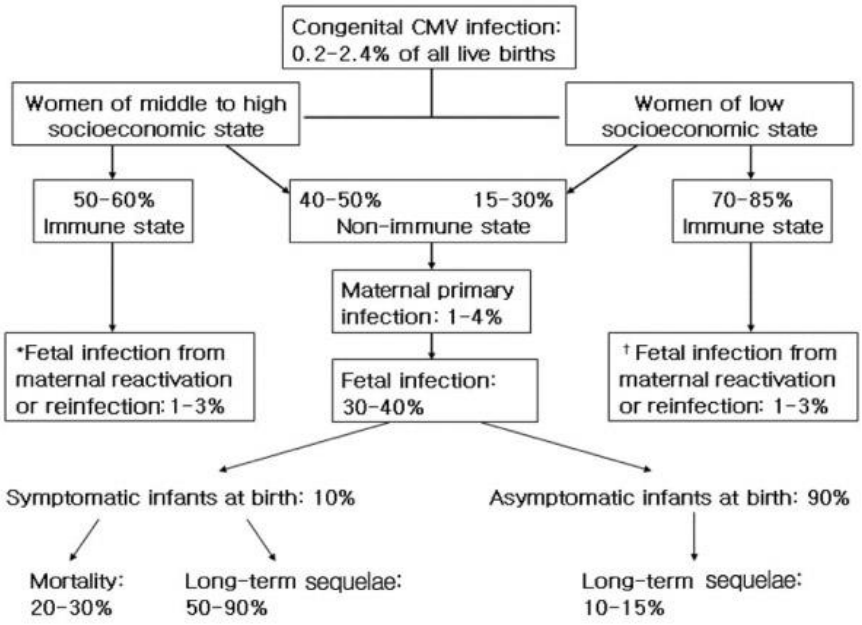


Figure 1.3: A schema of congenital cytomegalovirus infection and its outcome. (Adapted from Chun Soo et al. (2010) [18]).

HCMV replication

The HCMV genome, representing the largest genome of the characterized herpesviruses, encodes functional proteins. The HCMV coding potential was recently further examined, revealing an even higher level of genome intricacy [19]. The virus coded proteins, either contained in infectious virion particles or expressed in the cell at different stages of infection, interact closely with the cellular machinery.

The Human Cytomegalovirus genome is enclosed within an icosahedral capsid. The capsid is embedded in a dense protein matrix called tegument which in turn is surrounded by an envelope of numerous viral glycoproteins (Figure 1.4). The

formation of the virion starts at the host-cell nucleus, commencing with the assembly of the spherical procapsid. Once the procapsids mature, they move out of the nucleus and are believed to undergo a process of envelopment following a de-envelopment as they go across the nuclear membrane. The tegument is then acquired at specialized compartments termed the tegusomes. The final assembly of the protein envelope is carried out at Golgi-derived cytoplasmic compartments, where the virus gains its lipid membrane as well as the glycoprotein envelope. The complete virion exits the cell through the exocytotic pathway [20].

The HCMV infected cells produce three distinct types of viral particles. The first type contains the abovementioned mature capsids, the non-infectious enveloped particles (NIEP) [21] and finally the non-replicating particles which are termed dense bodies (DB) [22]. The NIEPs comprise from the same envelope proteins that the mature particles have, without the viral genome. The dense bodies are unique among all viruses and although they are unable to replicate in the host, they trigger the host immune response.

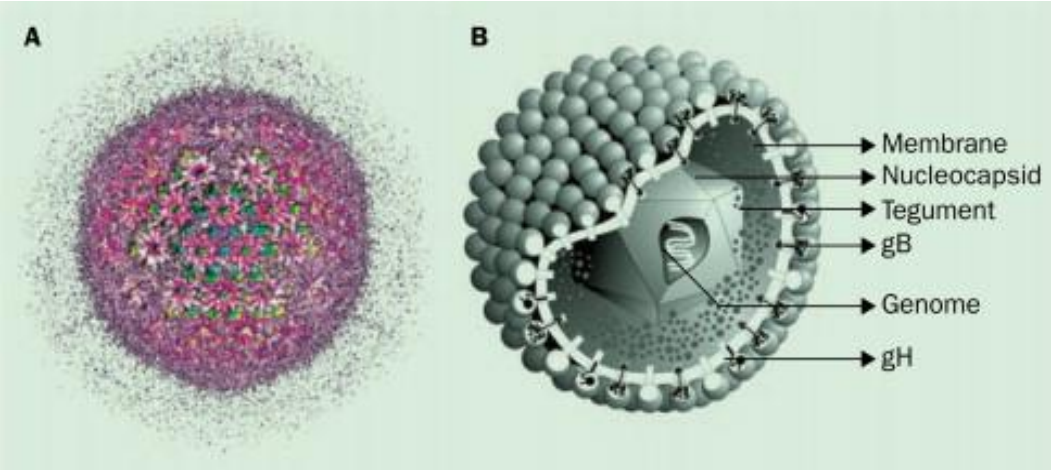


Figure 1.4: Human cytomegalovirus **(A)** Shaded surface representation of icosahedrally ordered portion of intact human cytomegalovirus particle. **(B)** Virtual three-dimensional model, showing various components of the cytomegalovirus. (Adapted from Maher K Gandhi et al. (2004) [23]).

HCMV life cycle

The viral life-cycle of HCMV is distinctively slower when compared to other herpesviruses, lasting for up to ten days in vitro. This is mostly due to the highly organized nature of the viral gene replication. The life cycle of HCMV comprises several stages: internalization, intracellular movement and intercellular spread and all of these processes are facilitated by interaction of viral components with cellular proteins. Among others, the actin cytoskeleton of the host cell and the cellular Rho GTPase signaling pathways that regulate the actin cytoskeleton are involved in several of these interactions [24].

During entry, viral envelope glycoproteins, positioned on the outside of infectious virions, interact with host receptors to mediate fusion or endocytosis of the virion into the cell (Figure 1.5 A) [25]. Viral tegument proteins bound to the capsid are believed to interact with the host microtubule machinery to transport viral capsids to the nuclear envelope and into nucleus (Figure 1.5 B), where viral transcription, genome replication and encapsidation occurs [26-28]. Simultaneously, other tegument proteins are deposited in infected cells by incoming virions and targeted to different subcellular locations to inhibit the initial steps of immune response and to regulate viral gene expression [29-31]. Furthermore, many viral-encoded proteins regulate cell-signaling pathways and cellular metabolism [32] to support viral replication and immune evasion. The expression of these viral proteins is a cascade of events which contains different genes regulating the virus life cycle (Figure 1.5; immediate early, IE; delayed early, DE; late, L) [33]. Capsids, assembled in the nucleus, egress through the nuclear double membrane by disruption of the nuclear lamina and formation of a nuclear egress complex (Figure 1.5 C) [34, 35]. Once

capsids reach the cytoplasm, the assembly and transport of virions occurs via the integration of multiple cellular trafficking pathways [36]. The endoplasmic reticulum (ER), Golgi apparatus, and endosomal machinery, is ''taken hostage'' for the formation of a cytoplasmic viral Assembly Complex (AC; Figure 1.5) [37, 38]. At the Assembly Complex, capsids acquire their tegument layer and viral envelope from intracellular vesicles. The generated infectious particles and the dense bodies, are next released into the extracellular space (Figure 1.5 D). Additionally, the host cell surface proteins play critical roles in HCMV entry and replication, as well as in host signaling and immune control [39].

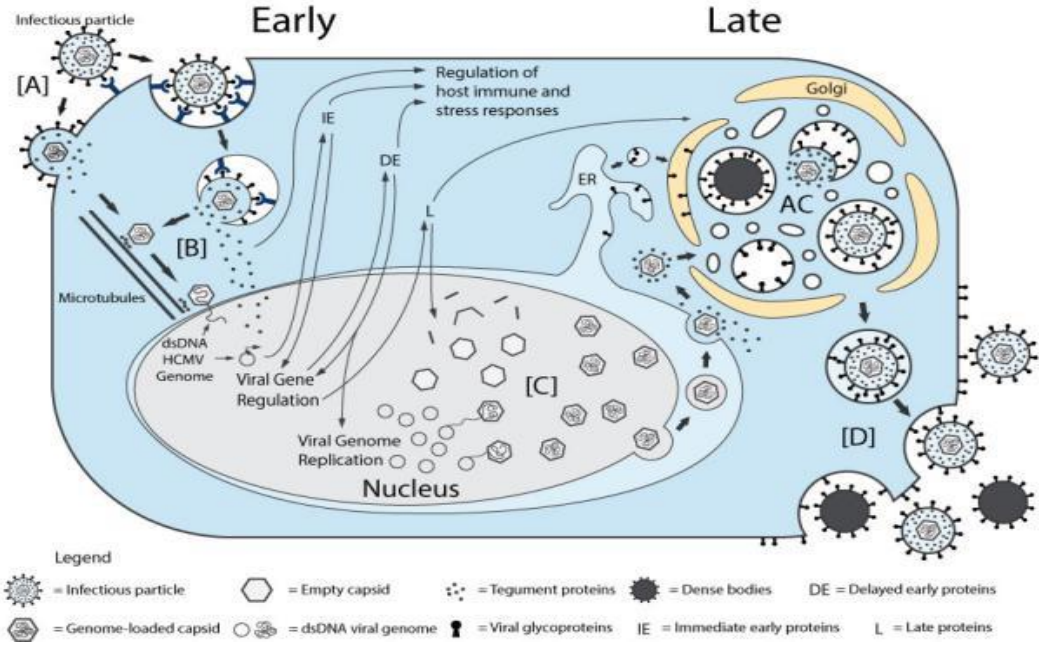


Figure 1.5: (A) Infectious particles enter the cell through interaction with cellular receptors. Capsid and tegument proteins are delivered to the cytosol. **(B)** The capsid travels to the nucleus, where the genome is delivered and circularized. Tegument proteins regulate host cell responses and initiate the temporal cascade of the expression of viral I immediate early (IE) genes, followed by delayed early (DE) genes, which initiate viral genome replication, and late (L) genes. **(C)** Late gene expression initiates capsid assembly in the nucleus, followed by nuclear egress to the cytosol. Capsids associate with tegument proteins in the cytosol and are trafficked to the viral assembly complex (AC) that contains components of the endoplasmic reticulum (ER), Golgi apparatus and endosomal machinery. The capsids further acquire tegument and viral envelope by budding into intracellular vesicles at the AC. **(D)** Enveloped infectious particles are released along with non-infectious dense bodies (Adapted from Beltran M Jean et al. (2014)[6]).

Virus-host interactions

Entry activated cell signaling

During the co-evolution with its host, HCMV has acquired a diverse range of mechanisms in order to abolish host signaling pathways and subsequently block host defense and gain viral replication. These interactions enclose protein to protein interactions and interactions between proteins and nucleic acids.

HCMV particularly infects quiescent cells and triggers their entry into G1 phase of the cell cycle in order to corrupt the cellular mechanisms that could be used for DNA synthesis. The molecular mechanisms by which HCMV sabotages host cellular functions, include altered RNA transcription, changes in the levels and activity of cyclin-dependent kinases, and other proteins involved in cell cycle control, post translational modifications of proteins, modulation of protein stability through targeted effects on the ubiquitin–proteasome degradation pathway, and movement of proteins to different cellular locations. When the cell is in the optimal G0 to G1 phase, many signaling pathways are altered and permit rapid induction of viral gene expression (Figure 1.6) [40].

At the early time of cytomegalovirus infection, immediate early gene regulation through the interaction of cellular factors with the major Immediate Early promoter (MIEP) and the MIE enhancer, as well as activation of early genes by IE proteins are rolling in [13]. Afterwards, cellular and viral proteins incorporate in the enhancer, the promoter and the viral elements in introns resulting gene-splicing regulation.

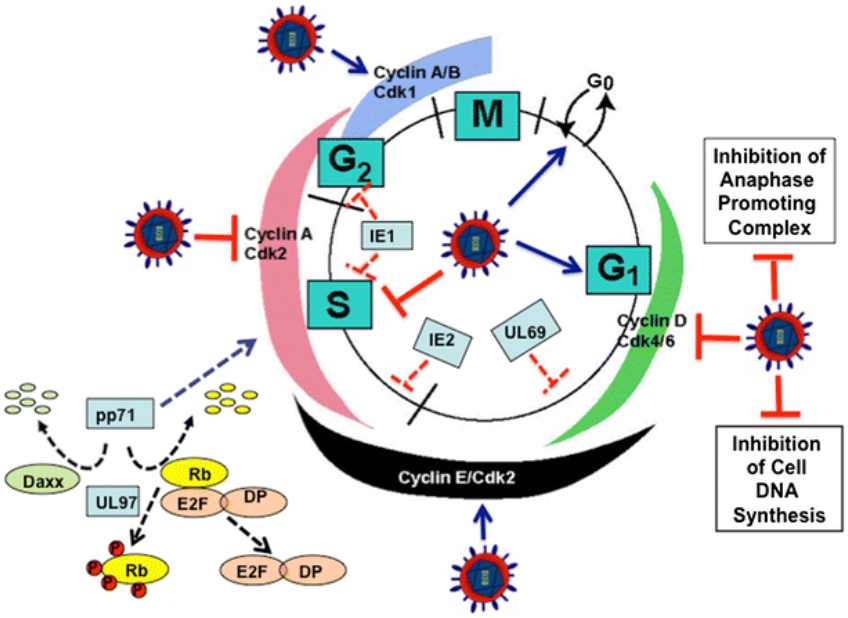


Figure 1.6: Cell cycle arrest in HCMV-infected cells is multifactorial. In uninfected cells, the anaphase-promoting complex (APC), an E3 ubiquitin ligase, remains active and targets proteins for degradation by the proteasome. HCMV infection of cells during G0/G1 phase induces progression through G1. (Adapted from Deborah H et al. (2015) [40]).

Glioblastoma stemness promotion

HCMV proteins have been identified in several types of solid tumors, such as malignant gliomas, neuroblastomas, medulloblastomas, prostate, colon, breast and lung cancer [41-44]. In more than 50% of these tumors, HCMV proteins are highly expressed in tumor tissues. Due to this association between HCMV and cancer, an oncomodulatory role for HCMV has been proposed [45-47]. Stable expression of HCMV IE1 protein has been shown to differentially enhance or arrest glioblastoma cell growth and promote cell cycle entry and DNA synthesis, indicating that IE1 can modulate the oncogenic phenotype of human glioma cells (Figure 1.7) [48]. Furthermore, HCMV IE1 can promote glioblastoma stemness, and survival demonstrating a novel role of IE1 as potent driver for glioblastoma stem-like cells [49]. The expression of HCMV glycoprotein G has also been detected in

glioblastomas enhancing growth and invasiveness through the activation of PDGFRα [50]. Moreover, HCMV promotes neoplastic transformation and induces mucoepidermoid proliferation by activating oncogenic signaling pathways [51]. HCMV has been also shown to inhibit apoptosis by regulating the activation of transcription factor 5 (ATF5) signaling pathway in human malignant glioma cells [52]. Persistence of HCMV in malignant glioma cells may result in a minimal cytopathic effect and therefore, HCMV may be reactivated in latently infected glioma cells when cells are exposed to inflammatory stimuli [53]. HCMV encodes for gene products that regulate cellular pathways involved in mutagenesis, apoptosis and host antitumor immune responses as their sustained expression edges to glioma constitution [54].

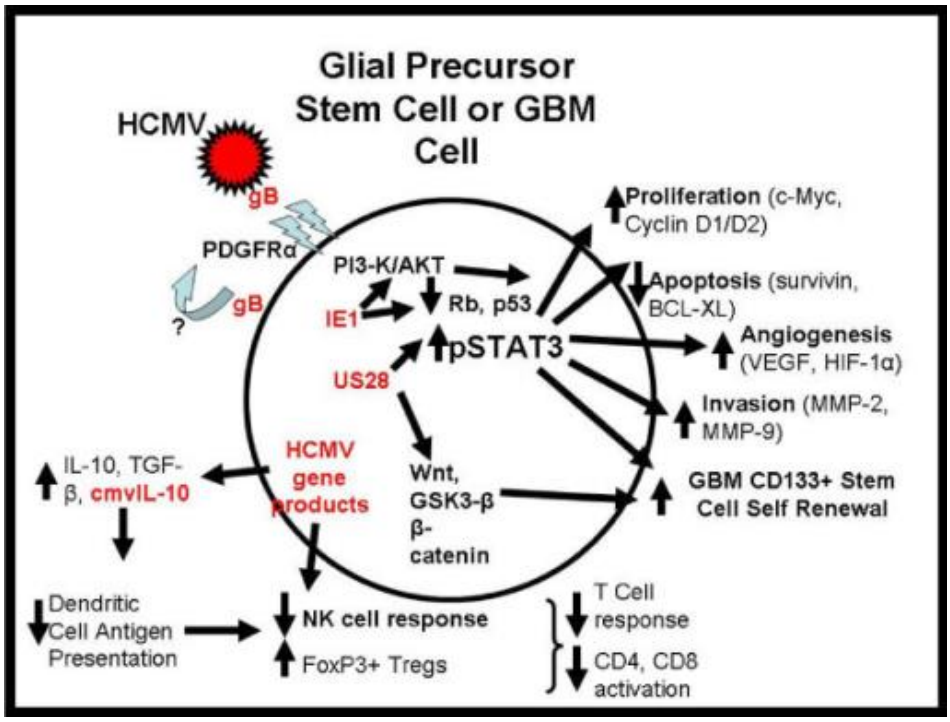


Figure 1.7: HCMV gene products encode for multiple proteins that can promote the various signaling pathways critical to tumor growth, including those involved in mitogenesis, mutagenesis, apoptosis, inflammation, angiogenesis, invasion and immuno-evasion. (Adapted from Buzdin AA et al. (2016) [55]).

Disease associations and therapy

HCMV propagation

Propagation of HCMV in cell culture has been an essential research tool but has limitations [56]. The commonly used laboratory strains have multiple mutations and deletions [57]. Remarkably, inactivating mutations in the RL13 gene are detectable by sequence analyses of the viral genome immediately upon propagation of virus in cell culture [58], which suggests that this gene is necessary for success of the virus in humans but strongly inhibitory to replication in cell culture.

HCMV antibodies

Antibodies in CMV-infected individuals have been useful for establishing serostatus. Although it has long been thought that only antibodies to gB or gH neutralize the virus, the finding that other viral genes, including UL128, UL130, and UL131A, mediate entry into endothelial and epithelial cells raises new possibilities for therapeutic design [59, 60].

Other disease associations

Various studies have shown that HCMV is reactivated in immunocompetent patients admitted to intensive care units, and there appears to be an association with prolonged hospital and ICU stay [61]. Moreover, associations of CMV with inflammatory diseases, diabetes, and tumors such as glioblastoma multiforme, as it mentioned above, have been suggested [62]. Furthermore, there are reports revealing that HCMV may play a role in immunosenescence and in the pathogenesis of atherosclerosis [63], possibly through actions of its many immunomodulatory genes.

Current therapies

Arresting strategies commonly target the early events of the replication cycle by using approved nucleoside analogs such as ganciclovir, the nucleotide analog cidofovir, and foscarnet. However, these can lead to resistance [64]. In vitro anti-sense oligonucleotides against the HCMV Immediate Early protein 2 (IE2) have proven effective [34], as well as targeting the UL36 and UL37 sites [65].

Ganciclovir, a guanosine analog, after phosphorylation by the HCMV UL97 kinase, acts as a chain terminator during viral DNA replication. The nucleoside monophosphate analog cidofovir and the pyrophosphate analog foscarnet which also inhibit viral DNA polymerase activity, but neither require prior activation by any other viral protein [66]. Ganciclovir products have been tested most widely in both transplant and HIV-infected subjects. In addition to oral formulations, ganciclovir can be given locally to the eye in patients with sight-threatening retinitis [67].

Experimental therapies

Several new anti-CMV compounds are presently in phase II clinical development. These include CMX001, a lipid derivative of cidofovir [68], and AIC246, which blocks a late step (possibly CMV terminase activity) in CMV replication [69]. The UL97 kinase inhibitor maribavir, even it has little serious toxicity, showed some efficacy in one controlled trial [70]. Donor derived CMV-specific T cell therapy has been also used in selected patients [71].

Prevention

The transmission patterns of CMV suggest several ways to prevent primary CMV acquisition. Transmission by sexual secretions can be prevented by use of condoms [72]. The risk of transmission via saliva can be reduced by handwashing and gloves [73]. Transmission via blood transfusion or organ transplantation is almost completely preventable by leukocyte reduction techniques applied to the blood products or by donor selection, respectively.

The development of vaccines has been a primary goal for controlling HCMV. Recent reports indicate that a subunit vaccine, consisting of CMV gB with MF59 adjuvant, reduces acquisition of CMV in seronegative mothers (Figure 1.8) [74]. The recent finding that rhesus macaques mount immune responses to antigens delivered by repeated sequential inoculation of rhesus CMV recombinants highlights a potential role for live CMV vaccine vectors, but also illustrates the challenges in developing a vaccine that can block CMV infection itself [75].

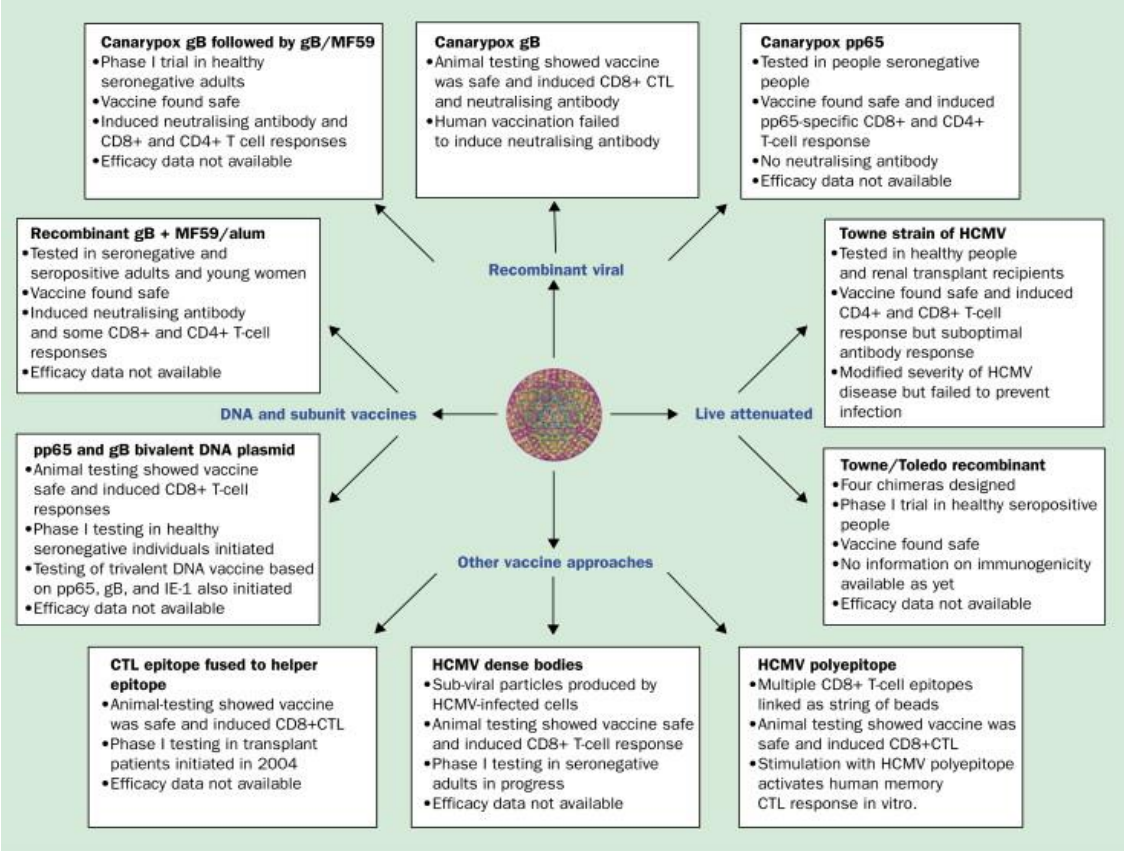


Figure 1.8: Outline of cytomegalovirus vaccine development strategies. (Adapted from Maher K Gandhi et al. (2004[23]).

Small Rho GTPases

General Information

The Rho GTPase family, a relative of Ras proto-oncogenes, consists of more than 20 proteins in humans, devided into subfamilies including Rho, Rac, Cdc42, RhoH, RhoBTB, Rho, Rnd and Rif (Rif and RhoD) [76]. Rho GTPases have been implicated in a variety of cellular processes and most importantly of cytoskeleton organization and their impact in biological functions, in cellular movement and division (Figure 1.9) [77, 78].

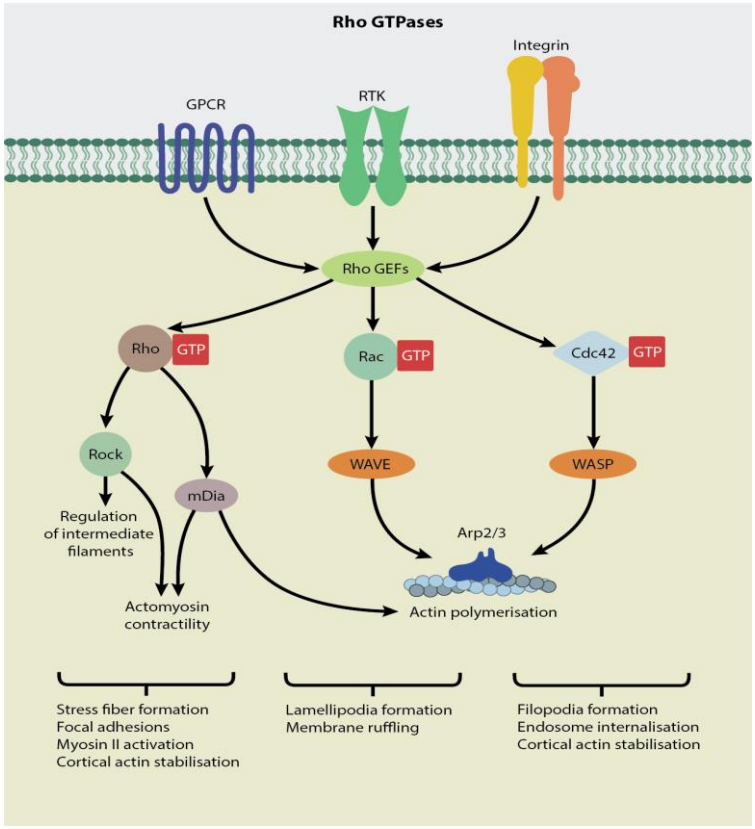


Figure 1.9: Rho GTPases regulate cytoskeleton dynamics. Rho family members are key regulators of actin reorganization and intermediate filaments (Adapted from MB Info: What are Rho GTPases?).

Cdc42, Rac1 and RhoA are the far well-characterized members of the superfamily. In addition, recent studies have shown that Cdc42, Rac1 and RhoA have a modulatory role in cellular trafficking and tumorigenesis [79]. Like other GTPases, the Rho GTPases cycle switches between the active GTP-bound form and inactive GDP-bound form. The guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) are the key regulators of the Rho GTPase superfamily [80]. GEFs mediate the dissociation of GDP and the recruitment of GTP, activating GTPases. GAPs facilitate Rho GTPase activation by stimulating Rho GTPases intrinsic GTP hydrolyzing activity, resulting in a

GDP-bound form. The third category of Rho GTPase family regulators, the Rho GDIs, retain the GTPases in an inactive form through their binding to C-terminal prenyl groups on Rho proteins (Figure 1.10) [81].

Through the interconversion between the inactive GDP-bound and active GTP-bound conformational states, Rho proteins are important molecular regulators of cellular functions such as cell proliferation, shape, polarity, adhesion and migration, vesicle trafficking, differentiation and transformation [82, 83].

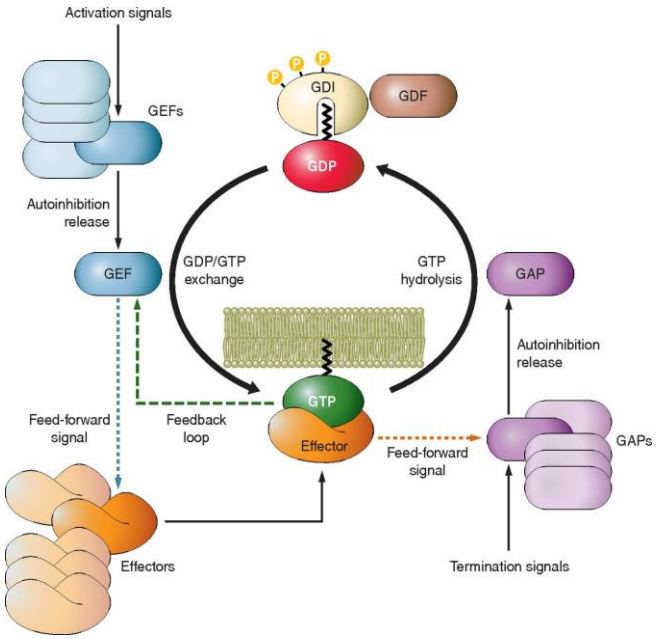


Figure 1.10: The basic GDP/GTP switch. Regulation of the GDP/GTP switch by GEFs, GAPs, and GDIs. All small GTPases are activated by GDP/GTP exchange stimulated by GEFs (blue) and inactivated by GTP hydrolysis stimulated by GAPs (violet). Several GTPases families also combine their GDP/GTP switch with a cytosol/membrane alternation regulated by GDIs or GDI-like proteins (beige). Each small GTPase subfamily is regulated by specific GEF and GAP subfamilies. (Adapted from Cherfils J et al. (2013) [84]).

Rho GTPase proteins and tumorigenesis

To establish metastasis in distant tissues, tumour cells have to enter the vascular or lymphatic system, then exit it and proliferate in a new tissue. The ability of Rho GTPases to regulate cytoskeletal dynamics, cell adhesion and cell migration [85] marks a central role for these proteins in cancer cell invasion and metastasis.

The highly conserved RhoA, RhoB and RhoC proteins are frequently aberrantly expressed in human tumours, with RhoA and RhoC being frequently overexpressed whereas RhoB is often downregulated [86]. RhoA has been implicated in all stages of cancer progression and has an important role during tumour cell proliferation, survival and progression, controlling the generation of epithelial polarity, junction assembly and disruption of epithelial cells [87].

Furthermore, RhoA is important for both amoeboid and mesenchymal migration through the activation of the RhoA-ROCK signaling pathway [88]. On the other hand, RhoC limits to metastasis in cancer progression. Studies of RhoC deficient mice show that RhoC is required for metastasis but not for the tumor initiation [89]. Besides, the expression of RhoB has been associated with tumor aggressiveness while it is often downregulated in human tumours [90]. RhoB has been proposed to act as a tumour suppressor inhibiting tumour growth, cell migration and invasion [90]. RhoB deficient mice have a normal developmental profile but display oncogenic formation [91].

Rho GTPase proteins and actin polymerization

These small Rho GTPases control the shape, mechanical and adhesive properties of fibroblasts [92]. Most notably, RhoA has been shown to induce assembly of focal adhesions and F-actin stress fibers, and to control the shape and adhesive and contractile properties of fibroblasts, as well as their capacity to organize the extracellular matrix (ECM) (Figure 1.11) [93, 94]. ROCK1, ARHGAP31, and ARHGAP26, have been shown to link with this Rho GTPase signaling pathway [95]. This link to the Rho pathway indicates that RhoA signaling pathway in fibroblasts, might mediate the control of tumor growth [96].

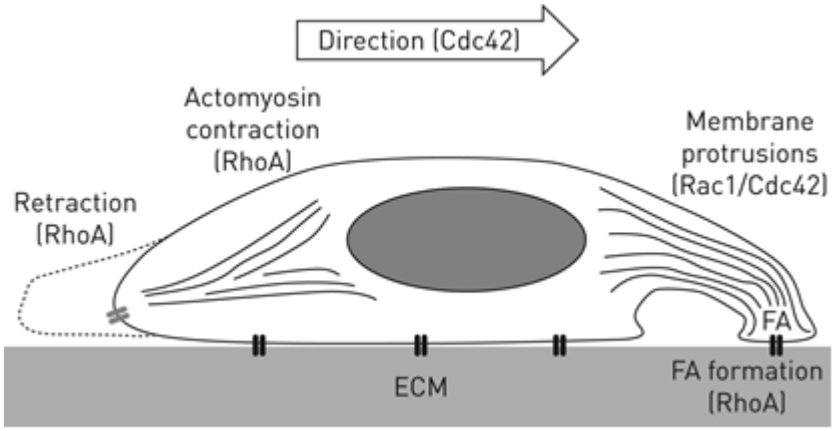


Figure 1.11: Cell migration is mediated by Rho-GTPase protein activity. Cell polarity is mediated by Cdc42 activity. Membrane protrusions are formed and are dependent on Rac1 (lamellipodia) and Cdc42 (filopodia) activity. Membrane protrusions grip the extracellular matrix via RhoA-dependent focal adhesion (FA) formation and actomyosin contraction pulls the cell forward via RhoA effector protein Rho-associated kinase (ROCK)-mediated phosphorylation of myosin light chain kinase. Disassembly of focal adhesion and tail retraction is also mediated by RhoA activity. ECM: extracellular matrix (Adapted from Millar Fr et al. (2017) [97]).

Cellular signals from extracellular environment activate RhoA in fibroblasts, which leads to increased expression and differentiation into myofibroblasts [98]. Consequently, tumor growth and invasion is shaped by cross-talk between

mechanical and biochemical signaling, which is modulated by RhoA signaling in fibroblasts (Figure 1.12) [99].

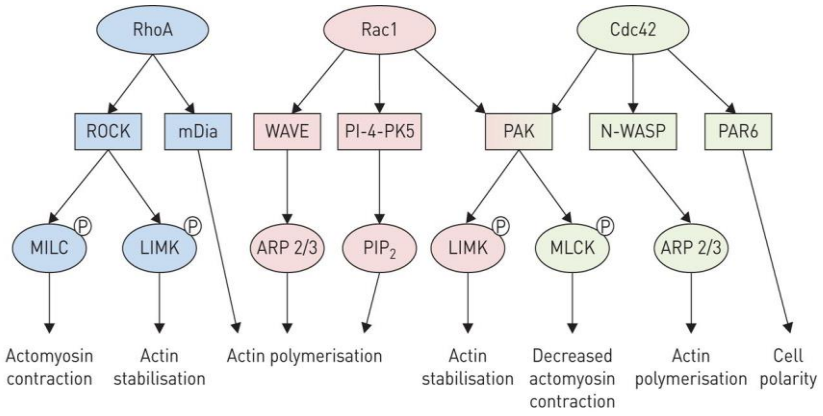


Figure 1.12: Rho-GTPases and downstream effector proteins involved in cell motility. RhoA acts via the effector proteins ROCK (RhoA effector protein Rho-associated kinase) and mDia to mediate actomyosin contraction and actin stabilisation. (Adapted from Millar Fr et al. (2017) [97]).

Herpesviruses and cytoskeleton

Herpesviruses and host nuclear architecture

Lamins are the best characterized cytoskeletal components of the cell nucleus that help to maintain the nuclear shape and participate in nuclear processes including replication and transcription. In addition, lamins have been shown to play a role in the propagation of viruses since nuclear lamina may represent a barrier for virions entering or exiting the nucleus [100].

Herpesviruses execute important steps of their replication cycles in the host cell nucleus. Among these steps is viral DNA synthesis, which occurs in discrete structures called replication compartments (RCs). Assembled capsids are then packaged with DNA before they translocate from the nucleus to the cytoplasm in a process called nuclear egress. To facilitate these steps, herpesviruses permit changes

to host nuclear architecture, including the formation and expansion of RCs, partitioning of host chromatin, and disruption of the nuclear lamina [101].

During infection with HCMV, viral DNA synthesis occurs away from the nuclear rim at the periphery of RCs [102-105]. The presence of capsid and terminase proteins in RCs suggests that capsid assembly and packaging are spatially coordinated with DNA synthesis within the nuclear interior [106-110]. Assembly and packaging are followed by nuclear egress [111, 112] and HCMV capsids move from RCs to the nuclear periphery for envelopment [113, 114].

Actin and Rho GTPase signaling in infected host cells

Nuclear actin is another cytoskeletal protein existing in the nucleus that achieves effective gene expression. Actin has been considered as a part of herpesviral virions. It can be incorporated into virions of human cytomegalovirus (HCMV) [115], murine cytomegalovirus (MCMV) [116], and Kaposi's sarcoma-associated herpesvirus (KSHV) [117]. Actin is localized predominantly in the tegument [118]. In the case of Pseudorabies Virus (PRV), it was shown that actin can partly replace the main tegument protein, VP22 [118]. Furthermore, filaments similar to F-actin are detecting in the perinuclear and extracellular virions of HSV-1 [119]. These filaments connect the nucleocapsid with the membrane envelope of the virion.

Throughout the years, human herpes viruses have employed genes encoding viral G protein-coupled receptors (vGPCRs), which are expressed in infected host cells (Figure 4.5). HCMV encodes four GPCRs [110] which have been modified to be used by the virus to take over the control of the host cell for its own benefit [111]. Rho GTPases which are activated through coupling of G proteins to GEFs in order to

stimulate proliferation, differentiation, and inflammation in a variety of cell and types [112], are also used from HCMV for the interaction of viral components with cellular proteins [113]. The engagement of viral proteins with the cell modulates the structure of the cytoskeleton actin and the function of actin effector molecules, such as Rho GTPases, to initiate infection and promote viral dissemination, which results in a high infection rate among the human population (Figures 1.13 and 1,14) [38, 114]. Furthermore, in the presence of HCMV, the knockdown of RhoB and RhoC proteins decreased the proliferation rate of glioblastoma, whereas the knockdown of RhoA protein in the HCMV infected cell lines restore their proliferation rate [120].

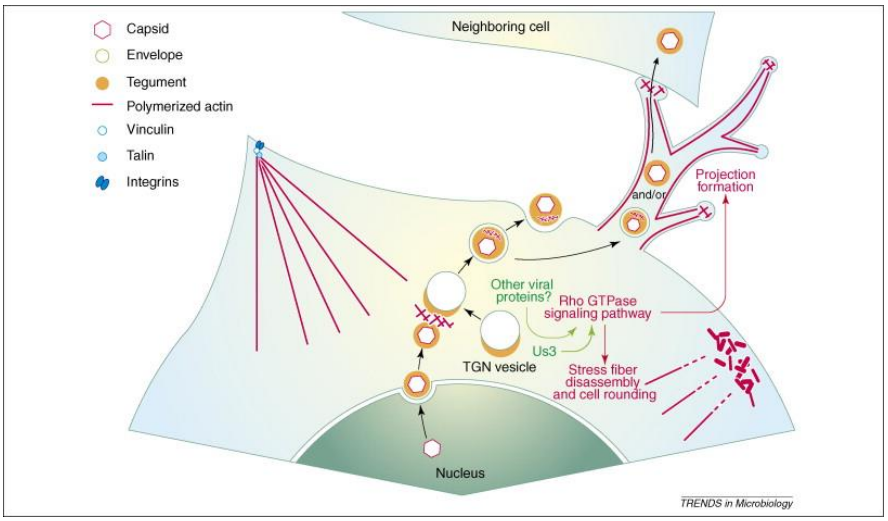


Figure 1.13: Human herpes viruses genes encoding viral G protein-coupled receptors (vGPCRs), which are expressed in infected host cells (Adapted from Favoreel H et al. (2007) [24]).

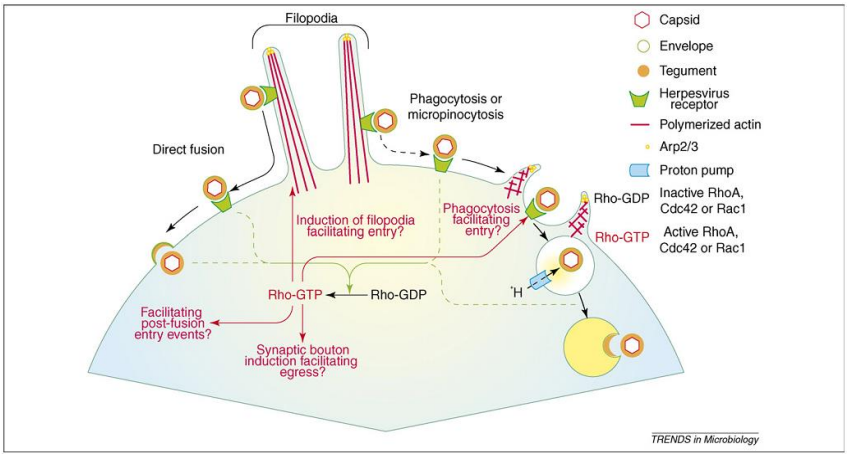


Figure 1.14: The implication of Rho family members in viral infection. (Adapted from Favoreel H et al. (2007) [113])

In conclusion, HCMV may modulate host cell cytoskeleton, proliferation rate and survival. These effects of HCMV infection arise from the interactions between viral regulatory proteins and Rho GTPase signaling pathway

2. Materials and Methods

Cells

Primary Human Foreskin Fibroblasts **(HFF)**, **HEK-293T** cells, the human glioblastoma cell line **U373MG** as well as its derivative **U373MG-IE1** cell line stably expressing the HCMV IE1 protein were grown in DMEM (Gibco BRL) supplemented with 10% foetal bovine serum (Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin under 5% CO_2 in a humidified incubator at 37°C. HFF cells were also used for the propagation and titration of HCMV virus. The stable expression of IE1 was maintained in U373MG-IE1 cells using G418 (1mg/ml) (Sigma-Aldrich, USA). Human embryonic kidney cell-line 293T (HEK-293T cells) was used only for the production of shRNA TRIPZ lentiviruses.

Viruses

The wild-type HCMV AD169 strain and the UL32-EGFP-HCMV-TB4051 (kindly provided by C. Sinzger) were used in this study. The virus stocks were propagated and titrated on HFF cells according to standard protocols [121]. The UV-inactivated HCMV AD169 virus was produced after UV irradiation (254-nm wavelength) of the active virus stock for 30 min at a distance of 20 cm [122].

Virus propagation

Viral stocks are propagated in Human Foreskin Fibroblasts (HFF) cells and titrated by plaque assays. For this reason, 10^6 HFFs were plated in six-well plates ($100mm^2$) and then incubated for 24 hours. The next day the cells are infected by the laboratory strain AD169 of HCMV at a multiplicity of infection (MOI) 0.5. The cells are then incubated until they become enlarged and start to detach from the well

surface. The increase in size and the detachment of cells is an indication of active HCMV infection and is termed as the cytopathic effect (cpe). Once the cpe is excessive the cells are harvested using a cell scraper and stored in a 15mL Falcon tube. The suspension is subsequently centrifuged at 2,000rpm for 10 minutes and at 4^{o}C. The resulting pellet contains the Cell Associated Virus (CAV) and is resuspended in 1mL of fresh medium. The suspension is then sonicated at high frequency for 3-4 minutes at 4^{o}C. Sonication is performed to cause cell rapture and release of the HCMV virions. Once sonication is finished, the suspension is centrifuged for 15 minutes at 13,000 rpm and the supernatant is collected.

The supernatant from the first centrifugation contains the Cell Released Virus (CRV), which is the virions that were circulating in the medium. The extraction of the HCMV virions is performed by spinning the suspension for 3 hours at 13,000rpm and at 4^{o}C. After this long centrifugation the viral particles will have formed a pellet which we resuspend in fresh serum-free DMEM overnight. The resulting virus suspensions are mixed and filtrated through a 0.4μ filter to remove any bacteria and other contaminants, while dimethyl sulfoxide (DMSO) was added to 1%. The virus stock is then used for infections or stored at -80^{o}C for 4-6 months.

Titration of HCMV

Human foreskin Fibroblasts are used for the titration of the propagated virus, following well-established protocols. Specifically, 4x10^{5} HFF cells are plated in six-well plates and after 24 hours incubation, serial dilutions of the viral stock are prepared on ice. Dilutions are usually between 10^{-2} to 10^{-6}. Prior to the addition of the virus, DMEM is removed from the wells so that the cells are barely covered. The

virus is then added and the infection is allowed for 2 hours. During this period the 6-well plate is gently shaken every 10 minutes.

After the 2 hour period, the little medium that exists is discarded and replaced with a mix containing human serum and DMEM. The human serum is positive of IgG antibodies for HCMV and will block any subsequent infection through the medium. 30µl (1/100) of serum is added per 3 mL of inoculum. The cells are then allowed to incubate for 10 days at which time the plaques will have started to form. To stain the plaques, GIEMSA staining is performed. 500µl of undiluted GIEMSA is added to each well and is allowed to rest for 2 hours. The wells are then gently washed with tap water and air-dried upside down. Following Giemsa staining the plaques that developed were counted and the viral titer was estimated by this formula: X= (# of plaques)/d*V where X= the titer, d=dilution factor and V =the volume of diluted virus instilled on the cells.

Lentiviral Propagation and Transduction

HEK-293T cells were transfected using 10 µl Fugene 6 (Promega) with 3µg of either TRIPZshRhoA (RHS4852, Thermo Scientific), TRIPZshRhoB (V2THS_172671, Thermo Scientific) or TRIPZshRhoC (RHS4743, Thermo Scientific) vectors, 2 µg of pCMV-dR8.91 (Delta 8.9) plasmid and 1 µg of VSV-G, according to the manufacturer's protocol. The TRIPZ Inducible Lentiviral Empty Vector shRNA Control (RHS4750, Thermo Scientific) was used as a control lentiviral vector. The vectors above are engineered to be Tet-on and produce tightly regulated induction of shRNA expression in the presence of doxycycline (500 ng/ml). Additionally, in these vectors, turboRFP and shRNA are part of a single transcript, allowing the visual marking of

shRNA-expressing cells. Forty-eight hours after the transfection, the supernatants were harvested, filtered and stored at -80o C. The supernatants were applied either to primary Human Foreskin Fibroblasts (HFF) either to U373MG and U373MG-IE1 cells in the presence of 8 μg/ml Polybrene (Sigma-Aldrich, USA). Transduced cells were selected after 48 hours using puromycin (1 μg/ml) (Sigma-Aldrich, USA) and maintained in the presence of this antibiotic.

Inhibitors

Y-27632 (cyclohexanecarboxamide dihydrochloride monohydrateis), a Rho-kinase inhibitor was purchased from Sigma Chemicals (St. Louis, MO) and was dissolved in distilled water. Y27632 (stock solution 10mM) was used at a final concentration of 10 μM. Cells were treated with Y27632 for 1 hour in fresh medium before the infection with HCMV.

MTT assay

MTT assay was performed to determine the cell proliferation rate of both the parental U373MG and the derivative U373MG-IE1 cells after the knockdown of RhoA, RhoB or RhoC proteins, either in the presence of absence of HCMV infection. Briefly, 2×10^6 cells were transduced with either the shRNA Empty Vector or the TRIPZshRhoA or the TRIPZshRhoB or the TRIPZshRhoC lentiviruses, induced with doxycycline and subsequently were either infected with HCMV (MOI=3 pfu/cell) or mock infected to quantify their proliferation rate at 1 and 3 days after infection. In each cell type, the yellow tetrazolium MTT reagent (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (Sigma Alndrich, cat. no. R8755) was added and incubated for 4 hours at 37oC followed by the addition of 150 μl MTT solvent

(dimethyl sulfoxide-DMSO) for 15 minutes and finally the measurement of the absorbance at 590 nm with a reference filter of 620 nm. All experiments were performed in triplicates.

In vitro wound healing assay

U373MG and U373MG-IE1 cells were transduced either with the shRNA Empty Vector or TRIPZshRhoA, TRIPZshRhoB or TRIPZshRhoC lentiviruses and were grown until high confluency in 6-well plates. Twenty-four hours after plating, the cells were either infected with HCMV (MOI=3 pfu/cell) or mock infected and the monolayer were wounded with a pipet tip after 48 h post infection. Cell debris was removed by washing two times with serum-free medium and monolayer maintained in medium for 72 hours with or without HCMV. The wound closure was monitored at 0, 3, 6, 9, 12, 24, 48 and 72 h.p.i, using an epifluorescent Leica DMIRE2 microscope, equipped with a Leica DFC300FX digital camera. For each experimental point 8 fields photographed and the cells migrating inside 3 mm of wound were counted. Each experiment was carried out in triplicates and the cell motility was quantified by ImageJ 1.4.3.67 analysis (Launcher Symmetry Software).

Immunofluorescence Analysis

For immunofluorescence, 1×10^5 U373MG cells, transduced with the empty vector (EV) lentivirus vector and induced with doxycycline, were plated on glass coverslips in 24-well plates. The cells were subsequently infected with the recombinant HCMV AD169/IE2-EGFP virus expressing IE2 fused to the enhanced green fluorescent protein [123] at MOI =3 pfu/cell. The cells were fixed with formaldehyde (4%[vol/vol] in PBS containing 2% sucrose) and the nuclei were stained with DAPI. Fluorescent

images of were acquired with an epifluorescent Leica DMIRE2 microscope, equipped with a Leica DFC300FX digital camera.

For immunofluorescence, 1×10^5 HFF cells were plated on glass coverslips placed in 24-well plates. The conditioned medium was aspirated, and the cells were washed with PBS, fixed with formaldehyde (4% [vol/vol] in PBS containing 2% sucrose) and permeabilized with Permeabilization Solution (Cat. No 5115, Millipore). The coverslips were incubated for 1 h with primary antibodies diluted in PBS containing 1% (vol/vol) fetal bovine serum at room temperature and they were subsequently washed in the same buffer twice before incubation with the secondary antibodies. Incubation with the secondary antibodies was performed likewise. The primary antibody used were anti-HCMV pp65 (sc-71229) was purchased from Santa Cruz (Santa Cruz, CA). The secondary antibody used was Alexa Fluor 488 donkey anti-mouse (Cat. No A21202, Invitrogen). The nuclei were stained with DAPI (Cat. No T3605, Invitrogen) at a dilution of 1:1000 in PBS. Rhodamine (phalloidin R415) and fluorescein phalloidin (F432) were purchased from Molecular Probes (Eugene, OR).

Time Lapse Microscopy

In chambered coverglass units (Lab Tek, Thermo Scientific) 1×10^5 glioblastoma cells transduced with the appropriate shRNA lentivirus vectors knocking down RhoA, RhoB or RhoC were seeded and infected at MOI 3 pfu/cell with HCMV AD169 virus. Forty-eight hours after infection, the cells were transferred in humidified chamber on the microscope stage with 5% CO_2 at 37^OC. Still images from live cells were taken every 15 minutes for 5 hours using an epifluorescent Leica DMIRE2 microscope,

equipped with a Leica DFC300FX digital camera. Images were exported as TIFF files and were processed by Photoshop.

Morphometry

For cell shape analysis in glioblastoma cells were plated in two-well coverglass units chambers and bright field and fluorescent images of live cells were acquired with an epifluorescent Leica DMIRE2 microscope, equipped with a Leica DFC300FX digital camera. The total area was measured with Metamorph Cellprofiler Software (Genome Biology).

Fractionation

For the fractionation experiments, the cells were collected in PBS with 1 mM EDTA as described above and the pellet was incubated for 10 min in hypotonic lysis buffer (20 mM Hepes pH 7.6, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% NP40, 20% glycerol, 1 mM DTT and protease inhibitors) at 4°C. Following incubation with the hypotonic buffer, the nuclei were pelleted at 15,600 g for 4 min at 4°C and the supernatant was collected as the cytoplasmic fraction. The nuclei pellet was washed three times in hypotonic lysis buffer and subsequently the nuclei were incubated with hypertonic lysis buffer (20 mM Hepes pH 7.6, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% NP_40, 20% glycerol, 1 mM DTT and protease inhibitors) for 30 min at 4°C. The samples were then briefly sonicated (40% amplitude for 15 sec at 4°C), and centrifuged at 16,100 g for 20 min at 4 °C and the supernatant was kept as the nuclear fraction.

Immunoblotting

Protein extracts were analyzed by Western blot. For whole cell extracts, the cells were washed with cold PBS, collected with 1 mM EDTA in PBS and pelleted at 2500 g for 10 min. The cells were then incubated for 10 min at 4°C with M-PER Mammalian Protein Extraction Reagent (Cat. No 78503, Thermo Scientific) along with protease inhibitors (Cat. No 78415, Thermo Scientific) and phosphatase inhibitors (Cat. No 1862495, Thermo Scientific) vortexing mildly every 2 min. The cell extracts were then briefly sonicated (40% amplitude for 15 s at 4°C), centrifuged for 15 min at 14,000 g at 4°C and the supernatant was kept for analysis. All protein extracts were quantified with Cayman Protein Determination kit (Cat. No 704002, Cayman). From each sample, 40 μg of protein was boiled in SDS gel-loading buffer, separated by electrophoresis and transferred on nitrocellulose membranes. The membranes were subsequently blocked in TBS-T buffer with 5% (w/vol) dried non-fat milk and incubated overnight at 4 °C with the primary antibodies. The primary antibodies used for immunoblotting were anti-HCMV mouse IE1 (BS500) and mouse pUL44 (BS510), mouse anti-actin 1:5000 (Cat. No MAB1501, Millipore), rabbit anti-GAPDH 1:5000 (Cat. No MBC2501, Millipore), rabbit anti-RhoA 1:500 (Cat. No 2117BC, Santa Cruz), rabbit anti-RhoC 1:500 (Cat. No 3430BC, Santa Cruz), rabbit anti-LIMK1 1:1000 (Cat. No 3842BC, Santa Cruz), rabbit anti- phospho-LIMK1/2 1:500 (Cat. No 3841BC, Santa Cruz), rabbit anti-cofilin 1:1000 (Cat. No 5175BC, Santa Cruz) and rabbit anti-phospho-cofilin 1:500 (Cat. No 5474BC, Santa Cruz). All antibodies were diluted in TBS-0.1% Tween-20 (vol/vol) containing 1% (w/vol) dried non-fat milk. Following incubation with the primary antibodies, the membranes were thoroughly washed and incubated with the secondary antibodies for 1 hour at room temperature,

washed in TBS-T and developed using Luminata Forte Western HRP Substrate (Cat. No WBLUF0100, Millipore) either on film or by the ChemiDoc™ MP System (Cat. No 170-8280, Bio-Rad) with the Image Lab v5.0 software (Bio-Rad).

RhoA activation assay

Levels of RhoA-GTP were measured using colorimetric based G-LISA RhoA (BK124, Cytoskeleton) activation assay. For this assay, cell lysates were prepared, and the assay was performed following the protocols provided by the G-LISA kit manufacturer (G-LISA activation assays, Cytoskeleton, www.cytoskeleton.com). OD (490nm) was measured with an EL808 Ultra Microplate Reader (Bio-Tek Instruments). Absorbance units in each sample were expressed after subtraction of the background units measured in protein-free lysis buffer.

Quantitative-PCR

The Nanogen Q-CMV Real-Time PCR kit (Nanogen Advanced Diagnostics, Italy) was used to quantify the nuclear HCMV genomes in combination with the ABI 7500 Fast system (ABI). The primers for HCMV are specific for the exon 4 region of the HCMV MIEA gene (major immediate early antigen, HCMV UL123) while in parallel, a region of the human β globin gene is also amplified. The limit of detection of the assay is approximately 0.25log10 copies/ml. All reactions were performed in triplicate, following the manufacturer's instructions.

Plasmids

Plasmid	Details
TRIPZshRhoA	RHS4852, Thermo Scientific
TRIPZshRhoB	V2THS_172671, Thermo Scientific
TRIPZshRhoC	RHS4743, Thermo Scientific
shRNA Control	RHS4750, Thermo Scientific

Antibodies

Primary	Details	Used for
anti-HCMV pp65 protein	sc-71229 Santa Cruz	IF
anti-HCMV IE1 protein	BS500 [124]	IF,WB
anti-HCMV UL44 protein	BS550 [124]	WB
fluorescin phalloidin	7432, Molecular Probes	IF
mouse anti-actin	MAB1501, Millipore	WB
rabbit anti-RhoA	2117BC, Santa Cruz	WB
rabbit anti-GAPDH	MBC2501, Millipore	WB
rabbit anti-RhoC	3430BC, Santa Cruz	WB
rabbit anti-LIMK1	3842BC, Santa Cruz	WB

rabbit anti-pLIMK1/2	3841BC, Santa Cruz	WB
rabbit anti-cofilin	5175BC, Santa Cruz	WB
rabbit anti-pcofilin	5474BC, Santa Cruz	WB
anti-RhoA antibody	GL01A, Cytoskeleton	G-LISA
Secondary	**Details**	**Used for**
Alexa Fluor 488 donkey anti-mouse	A21202, Invitrogen	IF
Alexa Fluor 488 goat anti-rabbit	A11008, Invitrogen	IF
Goat anti mouse IgG, Peroxidase Conjugated, H+L	AP124P, Millipore	WB
Goat anti rabbit IgG, Horseradish Peroxidase Conjugated, H+L	AP132P, Millipore	WB
Secondary antibody horseradish peroxidase conjugate	GL02, Cytoskeleton	G-LISA

Buffers

DNA extraction buffer

- Phenol (Gibco BRL)
- Chloroform (Fluka)
- EtOH 70% & 100% (Merck)
- PBS: 0.8% NaCl, 0.02% KCl, 0.14% Na2HPO4, 0.02% KH2PO4, pH= 7.4.
- DNA Elution buffer (TE): 10mM Tris, pH=8, 1mM Na2EDTA.

Electrophoresis buffers

- Agarose gel electrophoresis buffer (0.5× TBE): 0.09M Tris-HCl, 0.09M Boric acid, 2.5mM EDTA, pH 8.3
- DNA loading buffer: 0.25% bromophenol blue, 0.25% xylene red and 40% glycerol
- Protein loading buffer 3×: 0.2 M Tris-HCl, 6% SDS, 6% Mercaptoethanol, 15% glycerol and 0.03 % bromophenol blue
- Separating or Resolving gel buffer: 1.5 M Tris-HCl, 4% SDS (w/v), pH 8.8
- Stacking gel buffer: 1M Tris-HCl, 1.6% SDS (w/v), pH 6.8
- Running buffer for western blots 10×: 0.25mM Tris base, 1.92mM glycine, 1% SDS (w/v), pH 8.3
- Transfer buffer 1x: 0.025mM Tris base, 0.192mM glycine, 20% methanol, pH 8.3
- Nitrocellulose wash buffer: TBS, 0.1% Tween (v/v)
- Chemilumminescence buffer (ECL): ChemiLucent Western blot detection system (Chemicon)

Immunofluorescence buffers

- Mounting Fluid – Every 10 ml vial contains Tris buffered glycerine, fluorescence enhancer and less than 0.1% sodium azide (Chemicon).
- Fixative solution - 50 ml formalin, 20 g sucrose, QS to 1l με PBS (pH 7.2 - 7.6) (Chemicon).
- Permeabilization Solution - 0.5% Nonidet P-40, 10% sucrose, 1% fetal bovine serum, 0.01% sodium azide σε PBS (Chemicon).

Bacterial transformation buffers

- L-Broth: 1% Tryptone, 0.5% Yeast Extract, 1% NaCl, 0.05% ampicillin, kanamycin or chloramphenicol.
- Glycerol stock solution of bacterial cells: 20% glycerol in L-Broth containing bacteria. 0.8ml L-Broth + 0.2 ml glycerol.
- L-Broth: 300ml H2O + 6gr LB.
- Agar: 300ml H2O + 6gr LB + 3.6gr LB agar.
- STET: 40 g 8% Sucrose, 25ml 5% Triton X-100, 125ml 50mM EDTA PH=8, 12.5ml 50mM Tris PH=8.
- Lysozyme: 10mg/1ml STET.
- DH5a competent cells.

3. Results

A. RhoA silencing confers advantage to early and late stages of HCMV life cycle.

RhoA specific knockdown in primary Human Foreskin Fibroblasts (HFFs).

In our experiments we used primary Human Foreskin Fibroblasts (HFFs) and we established HFFs which were devoid of RhoA. HFFs were transduced with the doxyxycline-inducible TRIPZ lentiviral vector expressing shRNA specifically targeting RhoA (shRhoA). A doxycycline-inducible TRIPZshRNA lentiviral empty vector (EV) was also used, serving as a negative shRNA control vector. The lentivirus tansduced cells were subsequently selected with puromycin. The efficiency of silencing for RhoA was determined by Western Blot in total cell extracts (Figure 3.1).

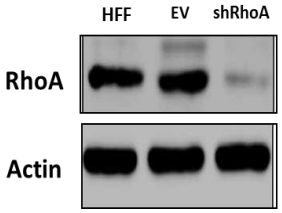


Figure 3.1: RhoA specific knockdown in primary Human Foreskin Fibroblasts (HFFs). The expression of RhoA was determined by Western Blot from whole protein extracts derived after transduction with the TRIPZ shRNA targeting RhoA lentivirus vector (shRhoA), induction with doxycycline and selection with puromycin. A doxycycline-inducible TRIPZshRNA lentiviral empty vector (EV) was also used, serving as a negative shRNA control vector. Actin served as loading control.

RhoA activity is reduced during the lytic infection of HCMV.

A complex protein network regulates GTPase cellular activity by switching them between GTP-bound active and GDP-bound inactive forms. The unique conformation of GTP-bound GTPases allows the activated GTPases to associate with downstream effectors that in turn carry out specific biologic responses. At the beginning, in order

to examine the effect of HCMV infection in the RhoA activity we used a recently developed RhoA activation assay. Cells were infected with the wild type AD169 HCMV strain or the UV inactivated HCMV at a multiplicity of infection 1 PFU/ml at different time points.

In uninfected control TRIPZshRNA lentiviral empty vector (EV) cells, RhoA activity increased, whereas RhoA depletion commenced a decrease in RhoA activity in the first 15 minutes with a further decrease of $\sim 20\%$ in 4 hours (Figure 3.2A). During HCMV infection, in control cells, RhoA activity was significantly decreased 2 hours after the infection, while at 4 hours of infection reached the ~30% of decrease (Figure 3.2B). Knockdown of RhoA did not alter the down-regulation of RhoA activity and interestingly displayed a greater decrease in RhoA activity at the early stages of infection compared to the control cells (Figure 3.2B).

Since data showed a consistently reduced RhoA activity in infected control and RhoA depleted cells, we went on to define whether the adhesion of virus alone is sufficient for this effect. For that reason, cells were infected with the incapable for active viral gene expression UV inactivated wild type HCMV AD169 at an MO1 1 PFU/cell at various time points. The UV inactivated virus had no effect in RhoA activity in control cells as well in RhoA knockdown cells (Figure 3.2C). The results mentioned above suggest that the adhesion of the virus alone is not sufficient, but active viral gene expression is required for the significant decrease of RhoA activity of the cells.

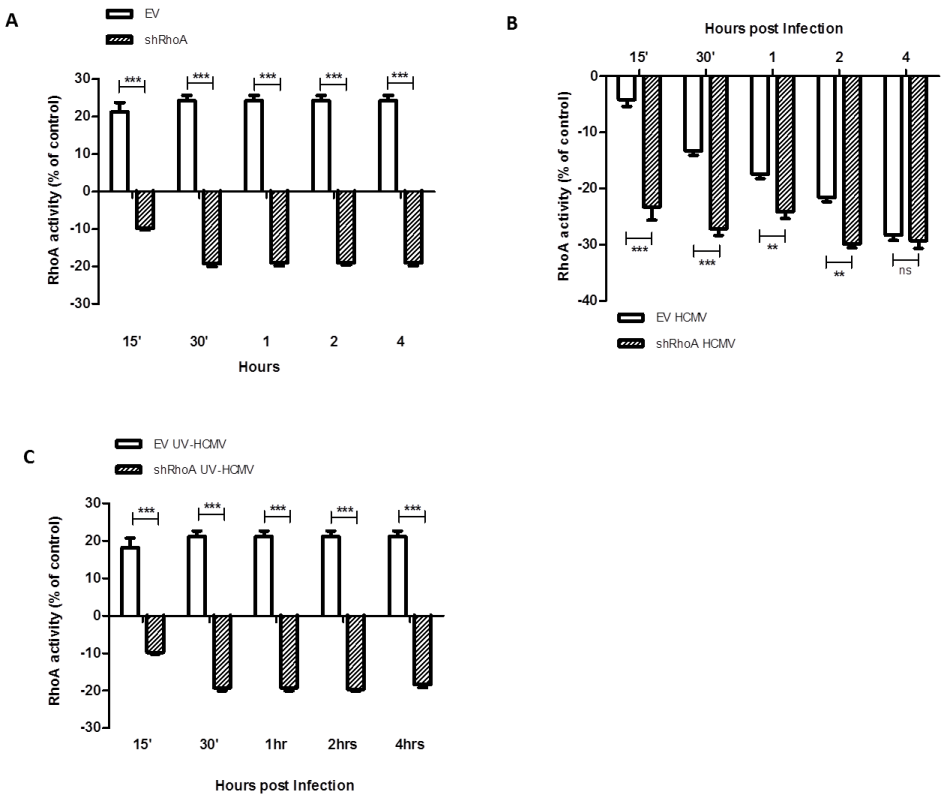


Figure 3.2: RhoA activity is reduced during the lytic infection of HCMV. (A) RhoA activity in mock EV and mock shRhoA depleted primary Human Foreskin Fibroblasts (HFFs). In transduced with the TRIPZshRNA lentiviral EV control cells RhoA activity increased, whereas RhoA depletion commenced a decrease in RhoA activity in the first 15 minutes. **(B)** RhoA activity in control and RhoA depleted HFFs infected with HCMV AD169 wt at a multiplicity of 1 PFU/cell at various points post infection. In control cells RhoA activity was significantly decreased 30 minutes after the infection. Knockdown of RhoA did not alter the down regulation pattern of RhoA activity and showed a range of ~30% decrease in RhoA activity during the HCMV infection time points. **(C)** RhoA activity in control and RhoA depleted HFFs infected with UV inactivated HCMV AD169 wt at a multiplicity of 1 PFU/cell at various points post infection. Infection of control and RhoA depleted cells the UV inactivated HCMV AD169 wt had no effect in RhoA activity. The cells lysates were collected and RhoA activity was quantified by G-LISA assay. For the statistical analysis, a Two-way ANOVA was performed using GraphPad Prism 6.04. The asterisks indicate statistical significance. ** indicate P = 0.001 to 0.01, *** indicate P = 0.0001 to 0.001. Data shown are means ± SD of five independent experiments.

RhoA depletion benefits production of HCMV progeny virions.

In light of the finding for consistently greater decrease in RhoA activity of infected RhoA depleted cells, we sought to inspect if silencing of RhoA benefits the production of HCMV virions. In this direction, control EV cells and RhoA depleted cells were infected with HCMV at MOI 0.1 PFU/cell for 2, 4 and 6 days respectively.

Titration assay showed that RhoA knockdown resulted in excessive production of progeny virions from as early as 2 days after the infection, compared to the control cells (Figure 3.3A).

The increase in HCMV progeny virions was also assessed at the level of viral protein synthesis. The expression levels of indicative HCMV proteins such as the immediate early protein IE1 as well as the early protein UL44 were examined by Western blot analysis. The infection of RhoA depleted cells by HCMV resulted in an increasing expression pattern for both viral proteins peaking on the sixth day post infection (Figure 3.3B).

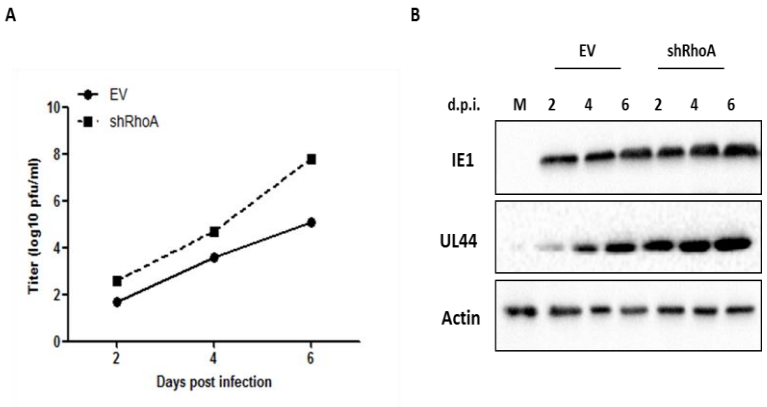


Figure 3.3: HCMV titer increases in RhoA depleted cells. (A) Growth curve of HCMV in control and RhoA knockdown cells. The cells were infected with HCMV ADT169 wt at MOI 0.1 PFU/cell and supernatants were harvested 2, 4 and 6 days post infection and they were titrated on primary Human Foreskin Fibroblasts. HCMV titer was significantly increased ($P<0.001$) in RhoA depleted cells. **(B)** IE1, UL44 and actin expression in control and RhoA depleted cells infected with HCMV AD169 wt at a multiplicity of 0.1 PFU per cell for 2, 4 and 6 hours respectively. Protein extracts were collected and analyzed by Western blotting. Analysis of statistical significance was performed by unpaired t-test (GraphPad Prism 6.04).

RhoA knockdown with subsequent ROCK1 inhibition benefits HCMV genome entry into nucleus.

The observed increase in the expression of HCMV proteins in RhoA knockdown fibroblasts prompted us to evaluate HCMV genome input in order to evaluate HCMV genome entry into the nucleus, compared to control cells. After the infection with the wild type HCMV AD169 strain at a multiplicity of infection 1 PFU/cell, the nuclear HCMV particles of the infected cells were quantified by real time PCR. The analysis revealed that the viral particle uptake of RhoA depleted cells was significantly increased when compared to the control cells as indicated by the amount of viral DNA (Figure 3.4A).

Based on the aforementioned finding which indicated that RhoA depletion gives advantage to HCMV genome entry in the host cell nucleus, we proceeded to investigate the RhoA downstream signaling pathway which is involved in this viral response. In particular, we explored the impact of ROCK1 kinase which is known to be activated by RhoA and involved in cofilin phosphorylation and thereby in force generation and contraction of cells. To address this issue, control and RhoA depleted cells were pretreated with the specific ROCK1 inhibitor Y27632 at a concentration of 10μM [125] and then infected with the wild type HCMV AD169 strain at MOI 1 PFU/cell. Subsequently the nuclear HCMV input of the infected cells was quantified by real time PCR. Interestingly, the number of viral input was significantly increased during the course of the infection and the pretreatment with the Y27632 inhibitor in TRIPZshRNA targeting RhoA cells when compared with the EV cells (Figure 3.4B). These data suggest that RhoA depletion with subsequent RhoA downstream effector ROCK1 inhibition benefit HCMV genome entry into the nucleus.

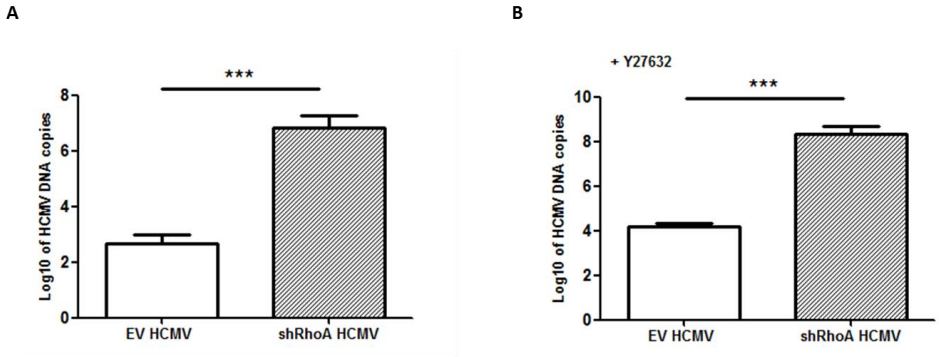


Figure 3.4: Effect of RhoA depletion and ROCK 1 inhibitor Y27632 in HCMV DNA input. (A) DNA copies of HCMV genome in control and RhoA depleted HFFs infected with HCMV AD169 wt at a multiplicity of 1 PFU/cell. **(B)** DNA copies of HCMV genome in control and RhoA depleted HFFs pretreated with Y27632 inhibitor (10µM) and infected with HCMV AD169 wt at a multiplicity of 1 PFU/cell. DNA extracts were collected and fractioned and HCMV genome copies were quantified by qPCR. For the statistical analysis, an unpaired t-test was performed using GraphPad Prism 6.04. The asterisks indicate statistical significance. *** indicate P = 0.0001 to 0.001. Data shown are means ± SD of three independent experiments.

ROCK1 inhibition benefits production of HCMV progeny virions.

To further confirm the involvement of RhoA and ROCK1 signaling pathway during HCMV infection we examined the production of progeny virions obtained from RhoA depleted and control cells infected at MOI 1 PFU/cell pretreated with Y27632, at 3 days post infection, compared to cells without Y27632 (Figure 3.5). The results showed a statistically significant difference in the production of progeny virions between RhoA knockdown cells with Y27632 and RhoA knockdown cells without Y27632. Similar results obtained for the control cells with/and without the Y27632 respectively. In addition, there was no statistically significant difference between the virus produced from the control infection with the Y27632 and the virus produced from RhoA depleted cells without the Y27632, a result which supports the role of

RhoA and ROCK1 pathway during HCMV infection confirming that either RhoA depletion or ROCK1 inhibition exhibit in similar effects during virus infection.

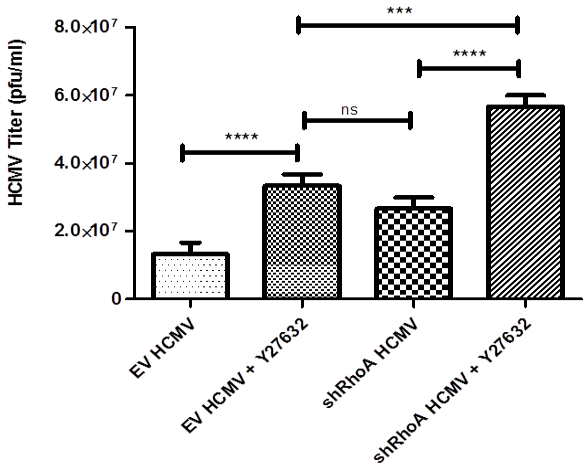


Figure 3.5: Effect of RhoA depletion and ROCK1 inhibitor Y27632 on HCMV progeny virus. shRhoA and control cells were pretreated or not with the Y27632 inhibitor. After pretreatment, the cells were infected at MOI 1 PFU/cell and the supernatants were collected at 3 days post infection and titrated in Human Foreskin Fibroblasts. Titrations are representative of three independent experiments and are shown as means ± SEM. For the statistical analysis, a Repeated Measures one-way ANOVA was performed and followed by an uncorrected Fisher's LSD test using GraphPad Prism 6.04. The asterisks indicate statistical significance. *** indicate p=0.0001 to 0.001 and **** indicate p<0.0001. ns= not significant.

RhoA depletion and HCMV infection alter the expression levels of ROCK1 downstream effectors.

Based on the evidence for implication of RhoA and ROCK1 pathway during the early and late stages of HCMV infection, we sought to investigate whether HCMV also impacts on the downstream effectors of this pathway. As mentioned earlier, RhoA is a multifunctional protein that, through the action of its various downstream partners, such as ROCK1, regulates actin cytoskeleton. Actin stress fibers formation downstream of RhoA activity is mediated by the RhoA effector ROCK1, which promotes myosin II phosphorylation and activation via inactivation of myosin light-

chain phosphatase (MLCP) and inactivates cofilin severing of polymerized actin filaments. Additionally, ROCK1 drives the phosphorylation of cofilin, through its interaction with LIMK, which promote stress fiber formation [126].

Since there are only few studies referring in the downstream effectors of RhoA ROCK1 signaling pathway during HCMV infection, we first investigated the pathway in primary Human Foreskin Fibroblasts (HFFs) and Western blot analysis was used to monitor the expression of LIMK1, phosphoLIMK, cofilin and phosphocofilin proteins. HFFs were infected with HCMV AD169 wt at MOI 1 PFU/cell at different time points. In order to reassure efficient infection, the viral IE1 expression was determined (Figure 6.6B). At the early stages of infection, HFFs displayed a decrease in the phosphorylation status of LIMK1 (Figure 3.6A). In contrast, at the later stages of infection the expression level of phosphoLIMK1 was not affected (Figure 3.6B). An analogous expression pattern was observed in the levels of total LIMK1 (Figure 3.6A and 3.6B). Furthermore, HCMV infection yielded an enhancement in the phosphorylation of cofilin at the early stages of infection, as well at late stages (Figure 3.6A and 3.6B). The expression level of cofilin at all stages of infection was not affected significantly. These preliminary data revealed that HCMV promotes alterations at the LIMK1 and cofilin expression levels which are more profound in the early events of infection.

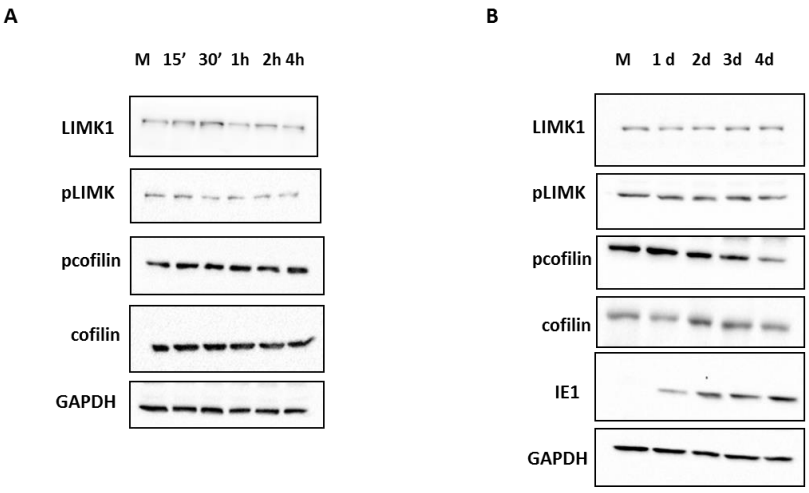


Figure 3.6: LIMK1, phosphoLIMK, phosphocofilin, cofilin and IE1 expression in primary Human Foreskin Fibroblasts (HFFs) infected with HCMV AD169 wt at a multiplicity of 1 PFU/cell, at various points post infection. (A) The cells were infected with HCMV AD169 wt for 15 and 30 minutes, 1, 2 and 4 hours respectively. **(B)** The cells were infected with HCMV AD169 wt for 1, 2, 3 and 4 days respectively. Protein extracts were collected and 40μg of protein were analyzed for each time point. GAPDH served as loading control.

Based on these preliminary data, we further examined the expression levels of the downstream effectors during the early stages of HCMV infection in RhoA depleted fibroblasts. The cells were infected with HCMV AD169 wt at MOI 1 PFU/cell at different time points. As it shown in Figure 3.7 during HCMV infection the phosphorylation of LIMK1 displayed reduction. Similar results observed for LIMK1 and cofilin expression levels. In contrast, the phosphorylation of cofilin showed important increase.

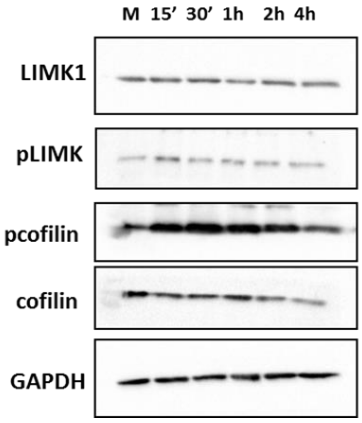


Figure 3.7: LIMK1, phosphoLIMK, phosphocofilin and cofilin expression in TRIPZshRNA targeting RhoA fibroblasts infected with HCMV AD169 wt at a multiplicity of 1 PFU/cell, at various points post infection. The RhoA depleted cells were infected with HCMV AD169 wt for 15 and 30 minutes, 1, 2 and 4 hours respectively. Protein extracts were collected and 40µg of protein were analyzed for each time point.

HCMV infection and RhoA depletion impair actin filaments organization.

Based on the aforementioned findings which pointed to the positive effect of RhoA depletion during HCMV infection, we further investigated the events associated with progression of viral infection by microscopy analysis. In order to monitor the effect of RhoA knockdown in actin filaments, control and RhoA depleted HFFs were examined under immunofluorescence microscopy. RhoA deficiency resulted in less regularly shaped cells compared with those of control fibroblasts. Furthermore, RhoA knockdown fibroblasts showed less formation of wide actin stress fibers. In addition, the structure of actin filaments in RhoA knockdown cells appeared less organized with very thin filament extensions (Figure 3.8Aand 3.8B).

While it is known that the impact of herpesviruses infection on the host cytoskeleton is immediate, and it commences with certain viral glycoproteins binding their cognate receptor on the surface of an uninfected cell, we sought to identify the

changes in actin cytoskeleton from the early stages of HCMV and host interaction. To that end, infected the HCMV AD169 wt at an MOI of 0.5 PFU/cell control and RhoA knockdown fibroblasts, were fixed 2 hours post infection and stained for FITC labeled phalloidin. Microscopy analysis showed that compared to control infected cells, RhoA depleted cells formed wider cellular projections with intense stress fibers (Figure 3.8A and 3.8B).

To further elucidate actin disruption we examined the effect of HCMV on actin network at late stages of infection. Infected control and RhoA knockdown fibroblasts were fixed 6 days post infection and stained for FITC labeled phalloidin. As shown in Figure 3.8A, the microfilaments probed with FITC labeled phalloidin, even they were depolymerized they could be readily observed in the infected control cells. However, the actin fluorescence in the infected RhoA knockdown cells was diffused and appeared in clumps suggesting a severe degradation of the actin network, especially at the egress sites (Figure 3.8B). Thus, this substantially destroy of actin filaments combined with the absence of RhoA, suggests a potential role of this protein in the process of enhancing the intracellular viral spread.

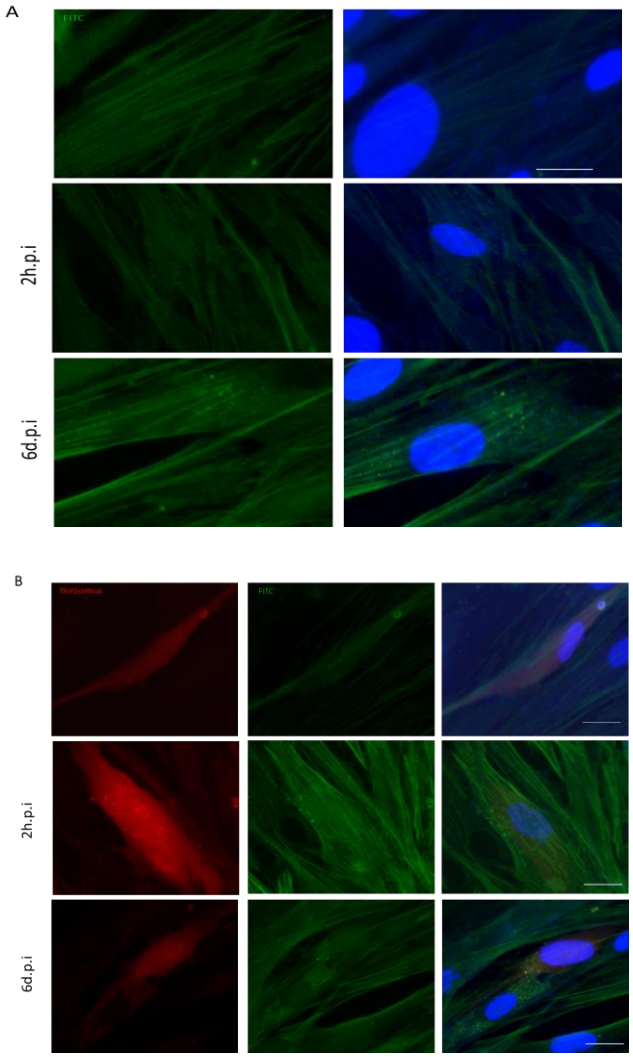


Figure 3.8: Analysis of the organization of the actin filament system as regulated by RhoA GTPase during the course of HCMV infection. (A) Cytoskeletal reorganization in mock and infected control cells. **(B)** Cytoskeletal reorganization in mock and infected RhoA depleted cells. TRIPZshRNA targeting RhoA cells exhibit red fluorescence. The cells were infected were infected with the HCMV AD169 wt at MOI 0.5 PFU/cell. Cells were fixed at the indicated time points after infection and actin fibers were visualized by direct immunofluorescence using FITC-labeled phalloidin. Nuclei were stained with DAPI. (scale bar 20 µm).

RhoA knockdown accelerates HCMV entry and egress from the cell.

Our results for the effect of HCMV in actin filaments of RhoA depleted cells in combination with previous studies [38] displaying a considerable role of RhoA during HCMV infection, prompted us to study the early and late events occurring after virus infection in the context of RhoA depletion. Control cells and cells which devoided RhoA, were infected with the recombinant virus UL32-EGFP-HCMV-TB40 expressing the capsid associated tegument protein pUL32 fused to EGFP at an MOI of 5 PFU/cell and fixed 2 hours post infection as well 6 days post infection. Immunofluorescence microscopy using this recombinant virus has proven that the EGFP signal represents true virion particles and does not originate from EGFP polypeptides while the kinetics and the proper localization of pUL32 by newly synthesized HCMV particles during the late stages of infection using this recombinant virus have been successfully shown in HFF infected cells [127]. However, 2 hours after the infection control cells displayed a delay in virus entry regarding the timeframe of expression of viral pUL32 protein, while in RhoA knockdown HFFs pUL32 protein was already accumulated alongside the nucleus (Figure 3.9A and 3.9B). Interestingly, the microscopic analysis at the late stages of infection, during the virus egress and in the absence of RhoA, revealed a greater number of EGFP foci which represent pUL32 containing newly synthesized virions and located in the cytoplasmic area between the virus Assembly Complex and the cell membrane, compared to the control cells (Figure 3.9A and 3.9B). In addition, in RhoA knockdown fibroblasts the pUL32 containing virions were increased in size and many of them were localized at a distance from the nucleus. The above observations provide evidence that depletion

of RhoA does not only accelerate HCMV entry, but even more maybe is associated with the effective propagation and viral egress.

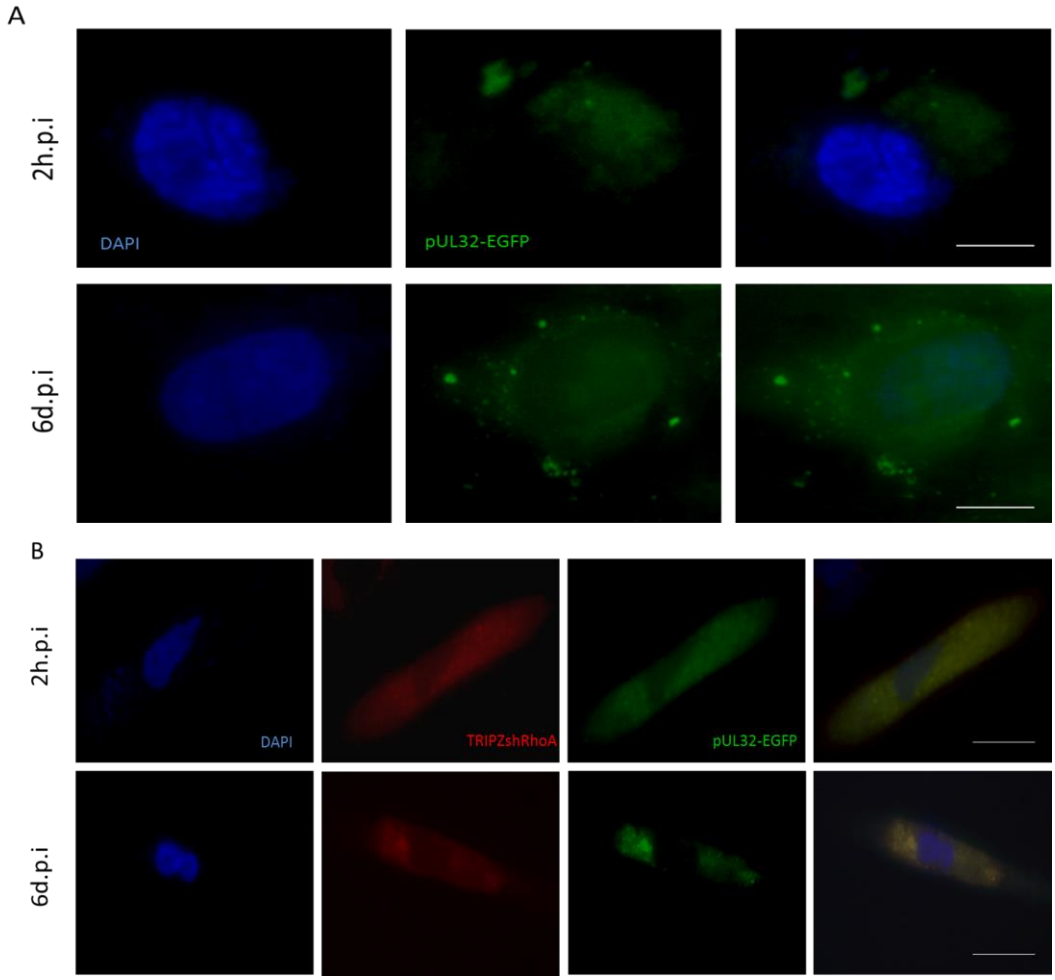


Figure 3.9: Visualization of the dynamics of RhoA GTPase during HCMV entry and egress from the cell. (A) The control cells were infected with the UL32-EGFP-HCMV-TB40 virus at MOI 5 PFU/cell and images were captured 2h.p.i and 6d.p.i. **(B)** The RhoA depleted cells were infected with the UL32-EGFP-HCMV-TB40 virus at MOI 5 PFU/cell and images were captured 2h.p.i and 5d.p.i. TRIPZshRNA targeting RhoA cells exhibit red fluorescence. Nuclei in fixed cells were stained with DAPI. (scale bar 20 µm).

RhoA depletion benefits HCMV Assembly Complex formation.

The effect of RhoA depletion during HCMV entry and egress from the cell, led us to investigate the role of RhoA in the intermediate stage of infection which contains the formation of virus Assembly Complex (AC). It is known that at late stages of infection, the AC becomes evident by the accumulation of viral proteins in the cytoplasm of the infected cell. In light of this, we examined the effect of RhoA depletion 3 days after the HCMV infection in fixed cells by immunofluorescence analysis. Infected cells were stained with the viral protein pp65 to define the cytoplasmic area that corresponded to the AC. Specifically, infected control cells and RhoA knockdown cells with an MOI of 0.5 PFU/cell were fixed and stained for pp65 protein 3 days after the infection and when the AC was formed. RhoA depleted fibroblasts displayed important changes in the abundance and localization of pp65 while in the infected control cells where the viral protein was also detected but not in the same pattern of distribution (Figure 3.10A and 3.10B). Compared to the restricted cytoplasmic distribution of pp65 in control cells, pp65 accumulated more profoundly at the cytoplasm in RhoA knockdown fibroblasts. At this stage of infection, pp65 was in a larger amount to the assembly site and localized at the AC (Figure 3.10A and 3.10B). The above observations provide evidence that depletion of RhoA accelerates the formation of viral AC during HCMV infection.

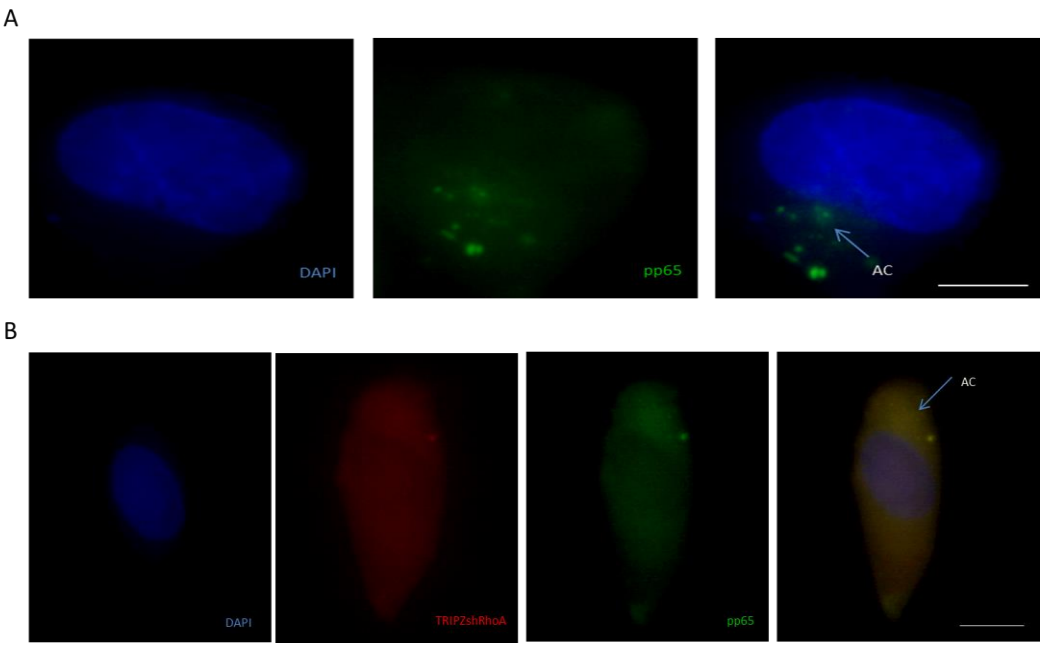


Figure 3.10: Visualization of the dynamics of Rho GTPases during HCMV assembly. The cells infected with HCMV AD169 at a multiplicity of 0.5 PFU/cell. **(A)** HCMV infected control cells were fixed 3 d.p.i. and they were immunostained for the viral AC marker pp65 (green). **(B)** HCMV infected RhoA knockdown cells were fixed 3 d.p.i. and they were immunostained for the viral AC marker pp65 (green). TRIPZshRNA targeting RhoA cells exhibit red fluorescence. Nuclei in fixed cells were stained with DAPI. (scale bar 20 μm).

B. The Role of RhoA, RhoB and RhoC GTPases in Cell Morphology, Proliferation and Migration in Human Cytomegalovirus (HCMV) Infected Glioblastoma Cells.

Generation of glioblastoma cells with stable knockdown of RhoA, RhoB and RhoC.

To investigate the role of RhoA, RhoB and RhoC in glioblastoma cells in the context of HCMV infection, we first established glioblastoma cells which were devoid of the above Rho GTPases. Parental U373MG cells and cells stably expressing the HCMV IE1 protein, named U373MG-IE1, were transduced with the doxyxycline-inducible TRIPZ lentiviral vectors expressing shRNA specifically targeting RhoA, RhoB or RhoC. A doxycycline-inducible TRIPZshRNA lentiviral empty vector (EV) was also used, serving

as a negative shRNA control vector. All lentivirus tansduced cells were subsequently selected with puromycin. The efficiency of silencing for each Rho GTPase was determined by Western Blot in total cell extracts (Figure 3.11A and 3.11B). RhoA or RhoC depletion did not affect the expression of the other isoform however, RhoA and to a lesser extent RhoC silencing induced RhoB overexpression, which is in agreement with previous studies [128, 129]. On the contrary, RhoB knockdown did not affect RhoA or RhoC expression levels.

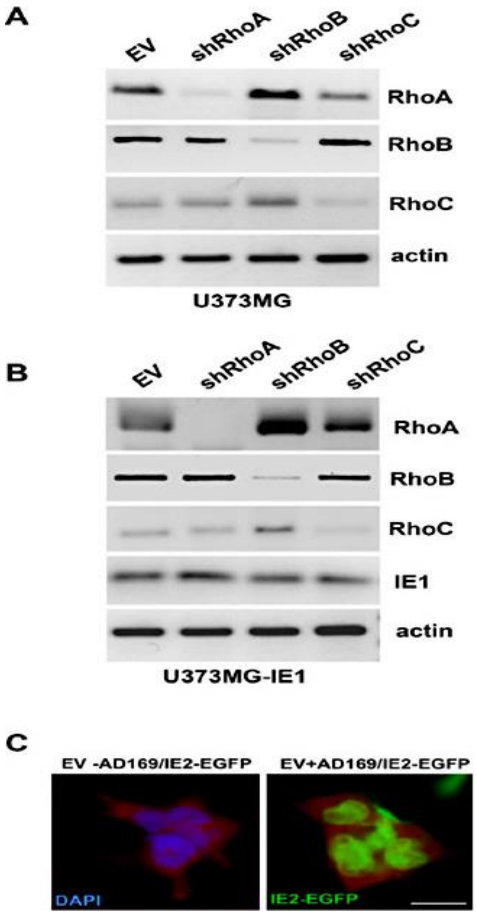


Figure 3.11: Rho isoform specific knockdown in U373MG and U373MG-IE1 cells. The expression of RhoA, RhoB and RhoC were determined in (A) U373MG cells and (B) U373MG-IE1 cells by Western Blot from whole protein extracts derived from each cell line after transduction with the indicated TRIPZ shRNA lentivirus vectors, induction with doxycycline and selection with puromycin. Actin served as loading control.The stable expression of IE1 in U373MG-IE1 cells was also confirmed. (C) U373MG cells, transduced with the lentivirus empty vector and induced with doxyxyclynine, were infected with the recombinant HCMV AD169/IE2-EGFP virus at MOI=3 pfu/cell. Cells were fixed 8 hours after infection and nuclei were stained with DAPI. (bar: 10 μm).

Cell morphology of RhoA, RhoB and RhoC knockdown glioblasoma cells in uninfected and HCMV infected cells.

We next examined the morphological changes caused by the knockdown of RhoA, RhoB and RhoC in glioblastoma cells, in the absence or presence of HCMV. In non-infected U373MG cells, depletion of RhoA resulted in an elongated cell shape compared to the control cells (Figure 3.12A). An analogous phenotype with long and thin protrusions was observed in RhoA-depleted U373MG-IE1 cells (Figure 3.12B). Similarly, RhoC-knockdown cells led to elongated, mesenchymal-like cells (Figure 3.12A and 3.12B), an observation which is consistent with the depletion of RhoC in other cell lines [60,61]. Increased cell spread was visualized in both U373MG and U373MG-IE1 uninfected cells when RhoB was silenced (Figure 3.12A and 3.12B).

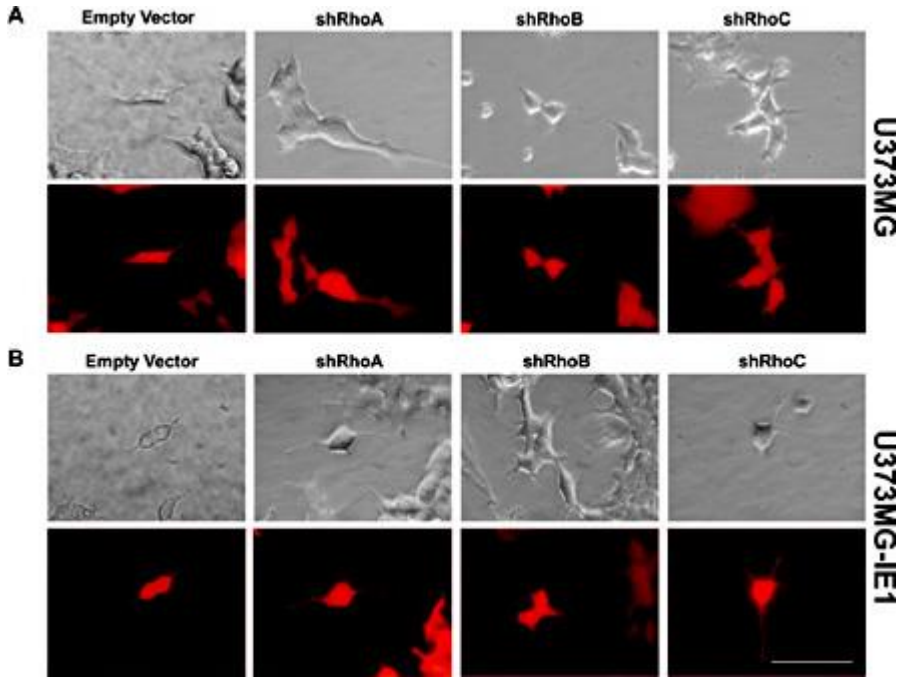


Figure 3.12: Differential morphological changes in RhoA, RhoB and RhoC-depleted glioblastoma cells. U373MG and U373MG-IE1 cells were transduced with the indicated TRIPZ shRNA lentivirus vectors, induced with doxycycline and selected with puromycin. Doxycycline-treated TRIPZshRNA-expressing cells exhibit red fluorescence. Still images were obtained by timelapse microscopy 48h after plating (bar: 50 µm).

In the context of HCMV infection, both U373MG and U373MG-IE1 RhoA and RhoC-depleted cells retained the basic feature observed in non-infected cells as described above, showing thin protrusions (Figure 3.13A and 3.13B). Interestingly, in the HCMV infected RhoB-knock-down cells, an increased number of cellular projections were formed, a phenomenon which has also been observed in primary HFF cells infected by HCMV [130]. Both HCMV infected glioblastoma cell lines with depleted each one of the three Rho isoforms were increased in volume, apparently due to the viral infection and the specific cytopathic effect HCMV causes.

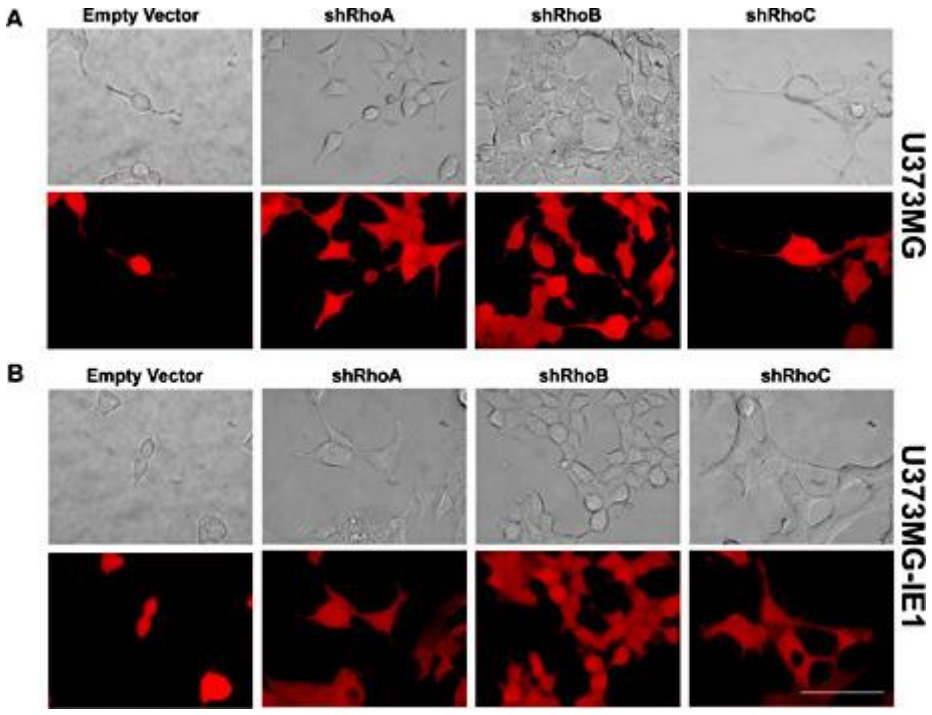


Figure 3.13: Phenotype changes of RhoA, RhoB and RhoC-depleted HCMV infected glioblastoma cells. U373MG and U373MG-IE1 cells were either mock infected or infected with HCMV AD169 (MOI=3 pfu/cell). Still images of live cells were captured 48h post infection (bar: 50 µm).

The changes in the shape between the control cells and the three Rho proteins knockdown tested, both in uninfected and infected U373MG and U373MG-IE1 cells were measured using the Metamorph CellProfiler software. The analysis statistically further confirmed the alterations visualized by microscopy (Figure 3.14).

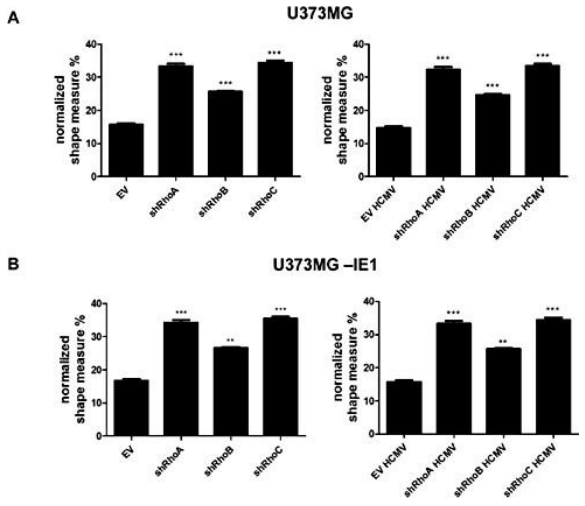


Figure 3.14: Cell shape changes in RhoA, RhoB and RhoC knockdown uninfected and HCMV infected glioblastoma cells. U373MG and U373MG-IE1 cells were transduced with the appropriate TRIPZ shRNA lentivirus vectors, induced with doxycycline and selected with puromycin. HCMV AD169 at an MOI=3 pfu/cell was added in a subset of cells. Still images were captured 48 hours after infection, processed and analysed by Metamorph CellProfiler. The asterisks indicate statistical significance. ** indicate P = 0.001 to 0.01, *** indicate P = 0.0001 to 0.001. Data shown are means ± SD of three independent experiments.

Effect of HCMV on the proliferation rate of U373MG and U373MG-IE1 RhoA, RhoB and RhoC knockdown cells.

We sought to determine the role of Rho GTPases in U373MG and U373MG-IE1 cells in uninfected and HCMV infected cells in terms of cell proliferation. Both cell lines were transduced with the appropriate shRNA lentivirus vectors knocking down RhoA, RhoB or RhoC, infected with HCMV AD169 when appropriate and subsequently tested in MTT assay. Depletion of either RhoB or RhoC in both the parental and the IE1 uninfected glioblastoma cells resulted in a significant inhibition

of the proliferation rate compared to the control (empty vector) cells (Figure 3.15A and 3.15B). Silencing of RhoA slowed down the cell growth of both parental and IE-1 derivative uninfected cells compared to the control cells but to a lesser extent compared to RhoB and RhoC silencing. HCMV infection alone did not affect the proliferation rate of either the U373MG or the U373MG-IE1 cells compared to the mock infected cells. Interestingly, HCMV infection of U373MG RhoA knockdown and U373MG-IE1 RhoA depleted cells did not cause a statistically significant difference compared to the control cells ($p = 0.1385$), whereas the proliferation state of the HCMV infected of RhoB or RhoC knockdown cells was inhibited ($p = 0.0941$ and $p = 0.0089$ respectively) (Figure 3.15A and 3.15B).

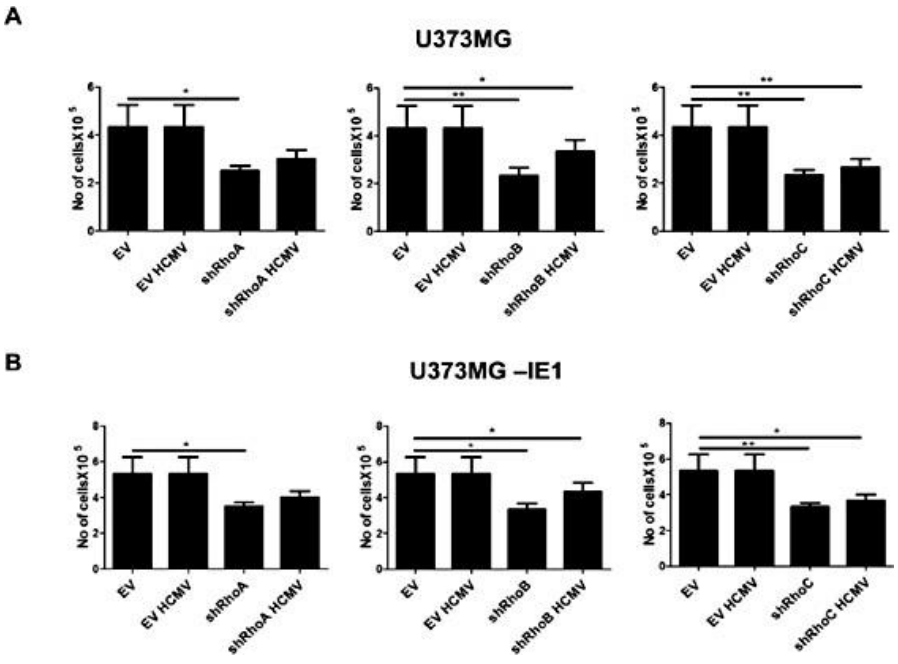


Figure 3.15: Proliferation rate of RhoA, RhoB and RhoC knockdown uninfected and HCMV infected glioblastoma cells. The cells were either mock infected or infected with HCMV at MOI=3 pfu/cell and 3 days after infection the cell proliferation rate was measured employing MTT Assay. The asterisks indicate statistical significance. * indicate P = 0.01 to 0.1, ** indicate P = 0.001 to 0.01. Data shown are means ± SD.

Depletion of RhoA, RhoB or RhoC inhibits random migration.

To characterize the function of RhoA, RhoB and RhoC in cell motility, we initially tested the migration of the corresponding free-moving Rho depleted U373MG and U373MG-IE1 cells (Supplementary movies: http://www.med.uoc.gr/research-lab-clinical-virology.php). Cells transduced with the empty vector often showed a long and polarized morphology whereas RhoA, RhoB and RhoC knockdown cells exhibited narrow lamellipodial structures at each projection and small dynamic protrusions along the elongated sides. When analyzing the route of individual cells from each Rho depleted cell type during 300 min migration period by timelapse microscopy, we found that the cells displayed a much shorter translocation than the control cells. Remarkably, the movement of RhoA silenced cells was dramatically limited, lacking their parental polarization and rather presenting an amoeboid fashion with cycles of expansion and contraction of the cell body. Infection with HCMV of the same cells did not cause significant changes in their motility and they rather showed reduced random migration speed. On the contrary, HCMV infected U373MG-IE1 cells exhibited a higher motility speed, enhancing the defect in the migration caused by the knockdown of each Rho protein and showing longer paths. Even in this more favorable context for the IE1-expressing glioblastoma cells, the free-movement of shRhoA cells was the most severely impaired compared to the control cells.

Rho GTPases are required for glioblastoma cell migration.

The role of RhoA, RhoB and RhoC in cell migration was explored in uninfected and HCMV infected parental U373MG and their derivative cells expressing the HCMV IE1 protein. Results showed that the knockdown of each of the three Rho isoforms

significantly decreased the average migration rate of U373MG cells compared to empty vector cells, as determined by wound healing assays. Moreover a differential reduction in the speed of cell movement was monitored, depending on which Rho protein was depleted. Among all three Rho proteins tested, the healing rate was considerably faster in RhoC knockdown cells, moderately slower in RhoB depleted cells while strikingly, the movement of RhoA silenced cells was significantly limited and they appeared almost stuck throughout the course of wound healing assay (Figure 3.16A). The aforementioned observations in Rho depleted cells did not change upon infection of the cells with HCMV (Figure 3.16B). The migration rate of U373MG-IE1 cells was also decreased in RhoA, RhoB and RhoC knockdown cells compared to the empty vector cells. However, this difference was remarkable compared to the parental cells and apparently, the expression of IE1 protein accelerates cell migration compared to the non-IE1 expressing cells (DATA NOT SHOWN). In contrast to Rho depleted parental glioblastoma cells, HCMV infection of the U373MG-IE1 cells after knockdown of RhoA, RhoB or RhoC restored the migratory behavior of the cells at comparable levels to the empty vector cells, either in the presence or absence of viral infection.

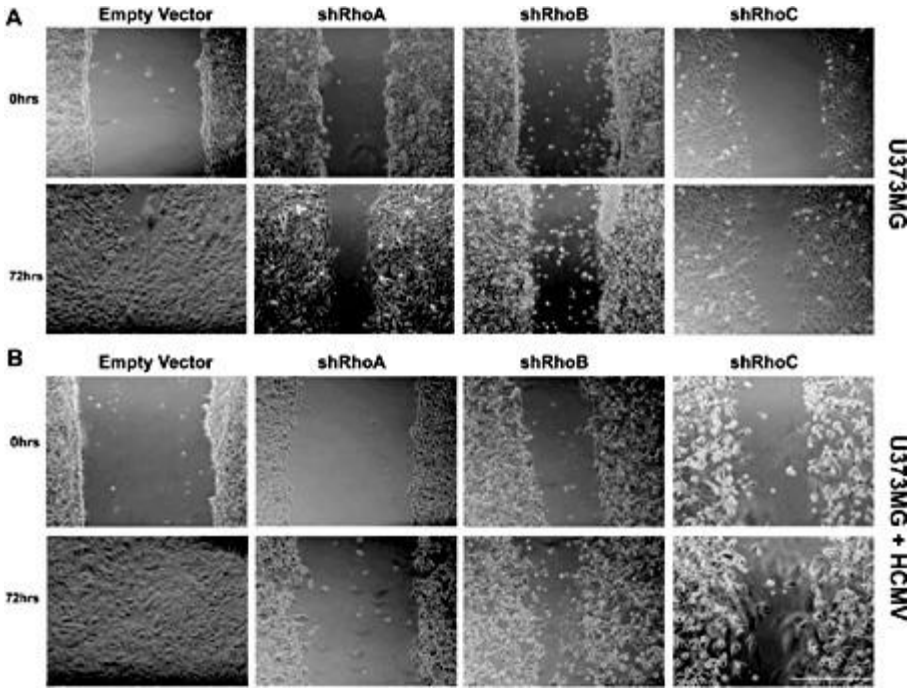


Figure 3.16: Effect of RhoA, RhoB and RhoC depletion on wound healing migration in uninfected and HCMV infected U373MG cells. U373MG were plated in 6-well plates and were either HCMV infected (MOI=3 pfu/cell) or not. The monolayer was "wounded" 48 hours post infection with a 10 µl sterile pipette tip and the detached cells were removed. Images were taken at 0, 3, 6, 9, 12, 24, 48 and 72 h after the scratch to monitor cell motility by timelapse microcopy. The initial (0 h) and final (72 h) time points of the assay are presented. (bar: 50 µm).

The area of the wounds was also recorded at different time points (3, 6, 9, 12, 24, 48, 72 hours) following the generation of the wounds. A marked delay in wound closure was observed in RhoA, RhoB or RhoC knockdown U373MG cells compared to the empty vector cells (Figure 3.17). Calculations on the area of the wounds demonstrated that in control cells, only 53% ± 1.9% of the initial wound area was left as opposed to all three Rho depleted cells where the wounds areas did not to change significantly, as only 80% ± 1.1% of them were covered. Close examination of cells at the wound edge revealed that control cells displayed a polarized phenotype, with cell protrusions perpendicular to the wound along with rapid elongation of cell projections. In contrast, cells with depleted Rho proteins exhibited a less evident

polarized phenotype, with RhoA knockdown manifesting a more drastic disruption of polarity, followed by RhoB and RhoC silencing. Although these cells could still form protrusions at the wound edge, their directions were more random and a substantial proportion of cells displayed multiple short protrusions or protrusions parallel to the wound.

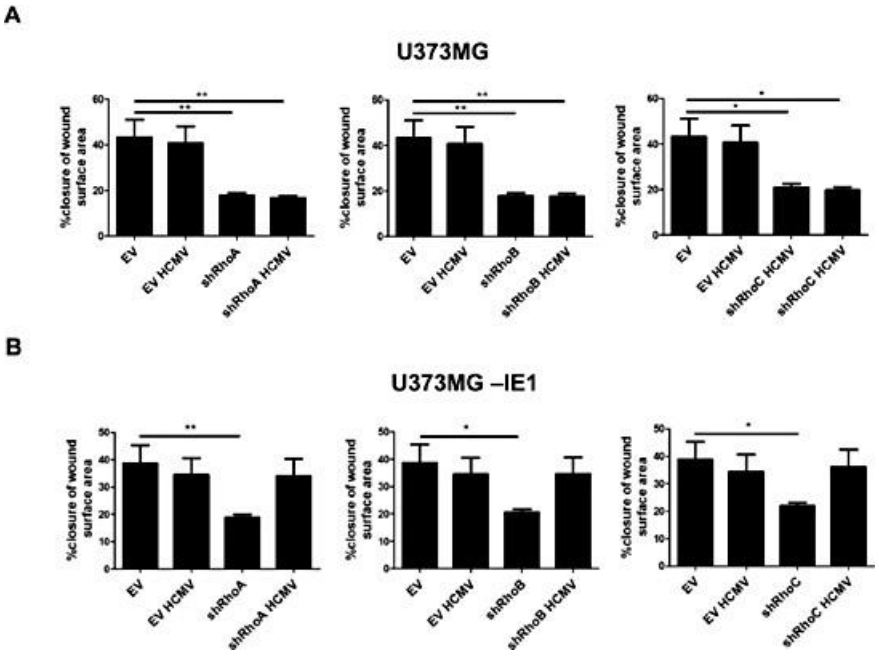


Figure 3.17: Quantification of wound healing assay in RhoA, RhoB and RhoC knockdown U373MG and U373MG-IE1 cells. Motility was quantified by measuring the decrease in the denuded area at 0 hours and 48 hours and presented as the average decrease in the number of pixels with standard deviation in three independent experiments. The asterisks indicate statistical significance. * indicate P = 0.01 to 0.1, ** indicate P = 0.001 to 0.01. Data shown are means ± SD of experiments in triplicate.

These phenotypes between the Rho depleted cells and the control cells remained unaltered during the course of HCMV infection. As regards the U373MG-IE1 cells, the defective closure capacity compared to the empty vector cells was also true when RhoA, RhoB or RhoC were silenced whereas HCMV infection of the same cells resulted in a remarkable increase in the closure efficiency of the wounds (Fig. 317B).

These data collectively indicate that RhoA, RhoB and RhoC play a favorable role in cell migration of glioblastoma overexpressing IE1 protein cells and also that HCMV confers a migration advantage to these cells in Rho proteins depleted cells.

4. Discussion

The role of Rho GTPases during Human Cytomegalovirus (HCMV) lytic infection was studied using two different approaches. Particularly, we studied the effect of inhibition of the host RhoA and ROCK1 signaling pathway on HCMV productive infection. Furthermore, we also studied the effect of silencing RhoA, RhoB or RhoC GTPases in cell morphology, proliferation and migration in HCMV infected glioblastoma cells.

A. RhoA silencing confers advantage to early and late stages of HCMV life cycle.

In this study, we demonstrate that RhoA depletion confers direct advantage in early and late stages of HCMV infection. This effect is specifically attributed to RhoA knockdown, as in early events of infection RhoA depleted cells displayed a greater decreased RhoA activity compared to the infected control cells (Figure 3.2B). We have also shown that the adhesion of the virus alone is not sufficient, but active viral gene expression is required for the significant decrease of RhoA activity of the cells (Figure 3.2C).

Furthermore, silencing of RhoA was found to benefit production of HCMV progeny virions (Figure 3.3A). In addition, the immediate early protein IE1 and early protein UL44 have been found to increase at 2 days after the infection as well as at 6 days post infection (Figure 3.3B). Congruent with these findings, the DNA input of the virus was significantly increased as early as 8 hours post infection in the nucleus of RhoA depleted fibroblasts (Figure 3.4A).

The aforementioned observations coupled with the reported downstream effectors of RhoA signaling pathway [131, 132] prompted us to investigate whether the RhoA downstream effector ROCK1 is implicated in this viral response. To that end, we explored the pharmacological Y27632 inhibitor which induces the downregulation of ROCK1. We observed that ROCK1 inhibition increased significantly the viral input in RhoA depleted cells compared to the control cells 8 hours after the infection (Figure 3.4B). Similar results were obtained when we evaluated the production of HCMV progeny virions in pretreated with ROCK1 inhibitor RhoA knockdown fibroblasts (Figure 3.5). Intriguingly, there was no significant difference between the virus produced from the control infection with ROCK1 inhibitor and the virus produced from RhoA depleted cells without the inhibitor, a result which supports the role of RhoA and ROCK1 pathway during HCMV infection and confirms that either RhoA depletion or ROCK1 inhibition exhibit in similar effects during virus infection (Figure 3.5).

Based on our original evidence for the implication of RhoA and ROCK1 pathway during the early and late stages of HCMV infection and due to the existence of only few studies referring in this pathway in the context of HCMV infection [133, 134], we further detected alterations in the expression levels of RhoA and ROCK1 downstream effectors during HCMV infection (Figure 3.6A and 3.6B). Particularly, our preliminary data revealed that HCMV promotes alterations at the RhoA and ROCK1 downstream effectors LIMK1 and cofilin expression levels which are more profound in the early events of infection (Figure 3.6A). In addition, we noticed for the first time that RhoA

depletion during HCMV infection caused more considerable alterations in expression levels of LIMK1 and cofilin (Figure 3.7).

In agreement with previous studies indicating that shortly upon HCMV infection, host actin filaments are disrupted and it has been described that there are 3 transient phases of actin depolymerization that occur at 20 minutes, 5 to 10 hours and 48 to 72 hours post infection [22, 109], our experiments revealed substantially destroy of actin filaments combined with the absence of RhoA (Figure 3.8A and 3.8B). These results suggest a potential role of RhoA in the process of enhancing the intracellular viral spread.

The important role of RhoA in HCMV life cycle was further illustrated by the critical accumulation of the viral protein pp65 at the Assembly Complex in HCMV infected RhoA depleted cells (Figure 3.10A and 3.10B). Very few studies have previously examined the role of RhoA during HCMV infection using specific inhibitors [107, 135]. However these studies focused mainly at the very early stages of HCMV infection. Rab genes, another family of small Ras like GTPases whose products control membrane traffic, are differentially regulated during HCMV infection have been shown to be essential for viral assembly and successful viral production [136]. After staining HCMV the infected RhoA knockdown fibroblasts with the pp65 viral Assembly Complex marker, we observed that compared to the restricted cytoplasmic distribution of pp65 in control cells, pp65 is accumulated more profoundly at the cytoplasm in RhoA knockdown fibroblasts. At this stage of infection, pp65 was in a larger amount to the assembly site and localized at the Assembly Complex (Figure 3.10A and 3.10B). This finding was further supported by microscopy analysis of cells

infected with the UL32-EGFP-HCMV-TB40 virus, which revealed an acceleration of pUL32 accumulation in the nucleus of RhoA depleted cells during the virus entry as well as a greater number of EGFP foci which represent pUL32 containing newly synthesized virions and located in the cytoplasmic area between the virus Assembly Complex and the cell membrane of these cells, compared to the control fibroblasts during HCMV egress (Figure 3.9A and 3.9B). The above observations provide evidence that depletion of RhoA accelerates virus entry and egress and the formation of viral Assembly Complex.

In conclusion, this study demonstrates for the first time a functional role of RhoA GTPase in HCMV life cycle. The finding that RhoA depletion and inhibition of RhoA downstream effector ROCK1 provide opportunistic effect in HCMV infection, raises the possibility that inhibition of RhoA and ROCK1 signaling pathway operates by disrupting vesicular trafficking within the infected cell and resulting in an acceleration in the trafficking of HCMV or its viral components to the nuclear membrane of the host.

B. The Role of RhoA, RhoB and RhoC GTPases in Cell Morphology, Proliferation and Migration in Human Cytomegalovirus (HCMV) Infected Glioblastoma Cells.

In this study, we demonstrated for the first time, an implication of Rho GTPases in cell morphology, proliferation and migration in human cytomegalovirus infected glioblastoma cells. Foremost, we observed that knockdown of RhoA resulted in an elongated cell shape with thin protrusions in non-infected and HCMV infected U373MG as well as in their derivative U373MG-IE1 cells. Likewise, depletion of RhoC led the cells to obtain an elongated, mesenchymal-like shape. The above

observations are consistent with earlier studies depleting the same Rho isoforms both in prostate and breast cancer cells [129, 137]. The overexpression of HCMV IE1 protein alone did not bring about any additional effect regarding the phenotype of the Rho knockdown cells, thus excluding any direct modulatory role of IE1 on cell morphology. The morphological changes induced in the glioblastoma cells after RhoA and RhoC depletion remained evident, even in the context of HCMV infection, despite the cytopathic effect caused by the virus, highlighting an imposing role Rho GTPases on cell shape. Although Rho GTPases have been shown to enhance malignant transformation and proliferation rate, RhoB is rather assumed as a negative regulator of these processes [91]. However, recent data have shown that RhoB expression is induced under conditions such as DNA damage, or treatment with growth factors or cytokines, suggesting that under particular circumstances, RhoB may favor towards human malignancy, including glioblastoma tumors [81]. In the present study, we show that when RhoB is silenced, both parental and their derivative-IE1 cells show a reduction in spread area compared to the RhoA or RhoC knockdown cells, an effect which has attributed to subsequent reduction of total surface levels of beta1 integrin [138]. Moreover, in the presence of HCMV, an increased number of cellular projections was formed, a phenomenon which has also been observed in HCMV infected primary HFF cells, particularly at the late stages of lytic infection [139]. These findings further corroborate the idea of a key role for RhoB in the regulation of cell shape.

Regarding to the proliferation rate of uninfected parental and IE1-expressing glioblastoma cells, the deficiency of RhoB or RhoC resulted in a significant inhibition

of their proliferation state while RhoA deficiency reduced the cell growth of both U373MG and U373MG-IE1 cells to a lower degree. This difference reflects the evidence that RhoA regulates actin polymerization, cell adhesion and myosin activity [140] whereas RhoB and RhoC control cell survival and cell proliferation, respectively [141]. This concept is furthermore supported by our observation that HCMV infection of U373MG and U373MG-IE1 RhoA depleted cells does not seem to affect significantly their proliferation rate, whereas the proliferation state of the HCMV infected of both parental and U373MG-IE1 RhoB or RhoC knockdown cells is statistically significantly inhibited. HCMV infection has been involved in human tumors influencing cell properties such as migration, invasion or cell signaling [142]. The results exploring the Rho GTPases silencing in association to cell proliferation of these cells and in the context of HCMV infection, indicate that RhoB and RhoC depletion in U373MG gliomablastoma cells rather results in the inhibition of the oncomodulatory effects of the virus. Interestingly, HCMV infection partially restored the growth rate of the RhoA knockdown glioblastoma cells, suggesting that the virus can differentially interfere with the proliferation state of the cells, depending on which Rho GTPase is depleted. This was also the case in the U373MG-IE1 RhoA knockdown cells. Apparently, the expression of IE1 alone and despite the oncogenic properties this protein may possess, it was not sufficient to confer a proliferation advantage to these cells and the active viral gene expression is required.

The alterations in the free movement of Rho knockdown cells were also monitored. The cells display diminished movement with U373MG and U373MG-IE1 depleted RhoA, RhoB or RhoC cells lacking their polarization and rather presenting an

amoeboid formation with cycles of expansion and contraction of the cell body reinforcing the evidence that Rho GTPases contribute to different features of cancer, such as invasion and metastasis [143]. Infection with HCMV does not change significantly their motility and they rather show reduced random migration speed. In contrast, HCMV infected U373MG-IE1 cells exhibit a higher motility rate, enhancing the defect in the migration caused by the deficiency of each Rho protein. It is worth noting the fact that even in the absence or presence of HCMV, parental and their derivative IE1-knockdown RhoA cells exhibited the lowest motility rate even in this more favorable for the IE1-expressing glioblastoma cells context, providing an additional proof reinforcing the significant role of RhoA to promote cell migration [144].

The aforementioned observations prompted us to investigate moreover the average migration of non-infected and infected U373MG depleted one of the three Rho isoforms cells, with RhoB and RhoC knockdown cells having the faster healing rate and in accordance with studies highlighting the role of these GTPases mainly in malignant transformation and to a less extend in motility, whereas the movement of RhoA silenced cells is decreased, revealing once more the differential role of RhoA, RhoB and RhoC in migration and invasion [145]. Interestingly, even if the migration rate of non-infected U373MG-IE1 depleted RhoA, RhoB and RhoC cells was also decreased, however, in the presence of HCMV, these cells partially restored their increased motility rate in migratory state, in contrast to the Rho depleted parental glioblastoma cells. This acceleration in cell migration could be due to the expression of viral genes, including IE1 protein since earlier studies have demonstrated that the

IE1 protein suppresses apoptosis and facilitates oncogenesis [146] and more recent studies that positively contributes to the replication cycle of HCMV [48].

Taken together, our data suggest an important implication of RhoA, RhoB and RhoC GTPases in morphology, proliferation and motility of both uninfected and HCMV infected U373MG glioblastoma cells. HCMV has been shown to manage signaling networks in infected cells, including the activation of G proteins [147] in order to reserve infection and viral spread. In this study, we display that downregulation of RhoA, RhoB and RhoC proteins in infected glioblastoma cells decreases their proliferation rate and their migration status and inhibits HCMV from worsening the malignant glioma pathogenesis. Interestingly, the presence of the HCMV Immediate-Early protein IE1 facilitates HCMV to retain its oncomodulatory advantage in these RhoA, RhoB and RhoC depleted cells. Therefore, we suggest a possible direct or indirect involvement of Rho small GTPases during HCMV infection with potential role in human glioblastoma cells.

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Appendix (Tseliou et al., 2016)

Cellular Physiology and Biochemistry

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Original Paper

The Role of RhoA, RhoB and RhoC GTPases in Cell Morphology, Proliferation and Migration in Human Cytomegalovirus (HCMV) Infected Glioblastoma Cells

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Key Words
HCMV • RhoA • RhoB • RhoC • Glioblastoma cells • Cell migration • Cell proliferation • IE1

Abstract
Background/Aims: Rho GTPases are crucial regulators of the actin cytoskeleton, membrane trafficking and cell signaling and their importance in cell migration and invasion is well-established. The human cytomegalovirus (HCMV) is a widespread pathogen responsible for generally asymptomatic and persistent infections in healthy people. Recent evidence indicates that HCMV gene products are expressed in over 90% of malignant type glioblastomas (GBM). In addition, the HCMV Immediate Early-1 protein (IE1) is expressed in >90% of tumors analyzed. ***Methods:*** RhoA, RhoB and RhoC were individually depleted in U373MG glioblastoma cells as well as U373MG cells stably expressing the HCMV IE1 protein (named U373MG-IE1 cells) shRNA lentivirus vectors. Cell proliferation assays, migration as well as wound-healing assays were performed in uninfected and HCMV-infected cells. ***Results:*** The depletion of RhoA, RhoB and RhoC protein resulted in significant alterations in the morphology of the uninfected cells, which were further enhanced by the cytopathic effect caused by HCMV. Furthermore, in the absence or presence of HCMV, the knockdown of RhoB and RhoC proteins decreased the proliferation rate of the parental and the IE1-expressing glioblastoma cells, whereas the knockdown of RhoA protein in the HCMV infected cell lines restored their proliferation rate. In addition, wound healing assays in U373MG cells revealed that depletion of RhoA, RhoB and RhoC differentially reduced their migration rate, even in the presence or the absence of HCMV. ***Conclusion:*** Collectively, these data show for the first time a differential implication of Rho GTPases in morphology, proliferation rate and motility of human glioblastoma cells during HCMV infection, further supporting an oncomodulatory role of HCMV depending on the Rho isoforms' state.

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Introduction

The Rho GTPase family, a relative of Ras proto-oncogenes, consists of more than 20 proteins in humans, devided into subfamilies including Rho, Rac, Cdc42, RhoH, RhoBTB, Rho, Rnd and Rif (Rif and RhoD) [1]. Rho GTPases have been implicated in a variety of cellular processes and most importantly of cytoskeleton organization and its impact on biological functions on cellular movement and division [2, 3]. Cdc42, Rac1 and RhoA are the far well-characterized members of the superfamily. In addition, recent studies have shown that Cdc42, Rac1 and RhoA have a modulatory role in cellular trafficking and tumorigenesis [4]. Like other GTPases, the Rho GTPases cycle switches between the active GTP-bound form and inactive GDP-bound form. The guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) are the key regulators of the Rho GTPase superfamily [5]. GEFs mediate the dissociation of GDP and the recruitment of GTP, activating GTPases. GAPs facilitate Rho GTPase activation by stimulating Rho GTPases intrinsic GTP hydrolyzing activity, resulting in a GDP-bound form. The third category of Rho GTPase family regulators, the Rho GDIs, retain the GTPases in an inactive form through their binding to C-terminal prenyl groups on Rho proteins [6]. Through the interconversion between the inactive GDP-bound and active GTP-bound conformational states, Rho proteins are important molecular regulators of cellular functions such as cell proliferation, shape, polarity, adhesion and migration, vesicle trafficking, differentiation and transformation [7-9].

To establish metastasis in distant tissues, tumor cells have to enter the vascular or lymphatic system, then exit it and proliferate in a new tissue. The ability of Rho GTPases to regulate cytoskeletal dynamics, cell adhesion and cell migration [10] marks a central role for these proteins in cancer cell invasion and metastasis. The highly conserved RhoA, RhoB and RhoC proteins are frequently aberrantly expressed in human tumours, with RhoA and RhoC being frequently overexpressed whereas RhoB is often downregulated [11]. RhoA has been implicated in all stages of cancer progression and has an important role during tumour cell proliferation, survival and progression, controlling the generation of epithelial polarity, junction assembly and disruption of epithelial cells [12]. Furthermore, RhoA is important for both amoeboid and mesenchymal migration through the activation of the RhoA-ROCK signaling pathway [13]. On the other hand, RhoC limits to metastasis in cancer progression [14]. Studies of RhoC deficient mice show that RhoC is required for metastasis but not for the tumour initiation [14]. Besides, the expression of RhoB has been associated with tumor aggressiveness while it is often downregulated in human tumours [15]. RhoB has been proposed to act as a tumour suppressor inhibiting tumour growth, cell migration and invasion [15]. RhoB deficient mice have a normal developmental profile but display oncogenic formation [16].

Human cytomegalovirus (HCMV) is a double-stranded DNA virus that belongs to the family of Human Herpesviruses. It is a beta herpes virus that establishes a lifelong latent infection after a primary infection [17]. HCMV has the largest genome among all herpes viruses, approximately 230 kbp that is divided into a unique large (UL) and unique short (US) region. HCMV encodes over 200 open reading frames, but the exact number is depending on the strain [18]. It is known for its opportunistic infection in immunocompromised individuals such as in AIDS patients, organ and stem cell transplant patients and it is the main etiological agent responsible for congenital diseases in newborn babies [19]. Replication of HCMV commences in the nucleus of host cells and is regulated by the expression of the immediate-early (IE) genes which are regulatory genes controlling early and late genes expression in infected cells [20]. HCMV IE genes 1 and 2 (IE1 and IE2) are the first set of viral genes that are activated in infected cells. IE1 and IE2 proteins regulate transcription of viral and cellular genes during HCMV infection and have been implied in the pathogenesis of many diseases [21].

HCMV proteins have been identified in several types of solid tumors, such as malignant gliomas, neuroblastomas, medulloblastomas, prostate, colon, breast and lung cancer [22-25]. Glioblastoma multiforme (GBM) is the most aggressive brain tumour in humans and is regulated by many different molecules [26, 27]. The microenvironment of glioblastomas seems to be restricted when the expression of KIF14 is inhibited [28] or due to low doses given of curcumin [29]. Furthermore, the tumour growth and progression of gliomas has been shown to be diminished in case of inhibition of autophagy in late stages [30]. In more than 50% of these tumours, HCMV proteins are highly expressed in tumor tissues. Due to this association between HCMV and cancer, an oncomodulatory role for HCMV has been proposed [31-33]. Stable expression of HCMV IE1 protein has been shown to differentially enhance or arrest glioblastoma cell growth and promote cell cycle entry and DNA synthesis, indicating that IE1 can modulate the oncogenic phenotype of human glioma cells and its expression in tumor cells activates signaling pathways associated with cancer. [34]. Furthermore, HCMV IE1 can promote glioblastoma stemness, cell-cycle progression and survival demonstrating a novel role of IE1 as potent driver for glioblastoma stem-like cells [35] and revealing that HCMV infection might promote pathogenesis in gliomas [36]. In addition, there are studies demonstrating that the IE1 expression increases the proliferation rate in primary glioblastoma cells via the suppression of p53 protein and the activation of Akt signaling [37-39] inducing the expression of a negative p53 protein regulator [40]. Beyond, the increase of telomerase activity is also correlated with IE1 gene expression in glioblastoma cell lines while there are reports displaying the co-localization of HCMV IE1 protein with hTERT proteins in gliomas [41]. Since it is well established that increased telomerase activity leads cells to be immortalized, this IE1 mediated enhancement of telomerase activity might modulate the microenvironment of tumour in glioblastoma cells [42].

The expression of HCMV glycoprotein G has also been detected in glioblastomas enhancing growth and invasiveness through the activation of PDGFRα [43]. Moreover, HCMV promotes neoplastic transformation [31] and induces mucoepidermoid proliferation by activating oncogenic signalling pathways [44]. HCMV has been also shown to inhibit apoptosis by regulating the activation of transcription factor 5 (ATF5) signaling pathway in human malignant glioma cells [45]. Persistence of HCMV in malignant glioma cells may result in a minimal cytopathic effect and therefore, HCMV may be reactivated in latently infected glioma cells when cells are exposed to inflammatory stimuli [46]. HCMV encodes for gene products that regulate cellular pathways involved in mutagenesis, apoptosis and host antitumour immune responses as their sustained expression edges to glioma constitution [47]. Throughout the years, human herpes viruses have employed genes encoding viral G protein-coupled receptors (vGPCRs), which are expressed in infected host cells. HCMV encodes four GPCRs [48] which have been modified to be used by the virus to take over the control of the host cell for its own benefit [49]. Rho GTPases which are activated through coupling of G proteins to GEFs in order to stimulate proliferation, differentiation, and inflammation in a variety of cell and types [50], are also used from HCMV for the interaction of viral components with cellular proteins. [51]. The engagement of viral proteins with the cell modulates the structure of the cytoskeleton actin and the function of actin effector molecules, such as Rho GTPases, to initiate infection and promote viral dissemination, which results in a high infection rate among the human population [52-55].

Given the growing interest in the role of CMV in cancer epidemiology, etiology, pathogenesis in combination with the active implication of Rho GTPases in human tumourigenesis, we aimed to investigate the role of Rho GTPases in HCMV permissive U373MG and their derivatives stably expressing IE1, U373MG-IE1 glioblastoma cells. Our results demonstrate an important role of RhoA, RhoB and RhoC in cell morphology both in the absence or presence of HCMV infection. Furthermore, the oncomodulatory role of HCMV was further revealed when the original inhibition in the proliferation rate of RhoA depleted cells was restored in the presence of viral infection.

Materials and Methods

Cells and virus

Primary Human Foreskin Fibroblasts (HFF), HEK-293T cells, the human glioblastoma cell line U373MG as well as its derivative U373MG-IE1 cell line stably expressing the HCMV IE1 protein were grown in DMEM (Gibco BRL) supplemented with 10% foetal bovine serum (Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin under 5% CO in a humidified incubator at 37°C. HFF cells were used for the propagation and titration of HCMV virus. The stable expression of IE1 was maintained in U373MG-IE1 cells using G418 (1mg/ml) (Sigma-Aldrich, USA). Human embryonic kidney cell-line 293T (HEK-293T cells) was used only for the production of shRNA TRIPZ lentiviruses. The wild-type HCMV AD169 strain was used in this study. The virus stocks were propagated and titrated on HFF cells according to standard protocols [56]. For viral infections, the cells were infected with HCMV at the indicated MOI for 2 hours and then the inoculum was removed and replaced by fresh medium.

Lentiviruses Production

HEK-293T cells were transfected using 10 μl Fugene 6 (Promega) with 3μg of either TRIPZshRhoA (RHS4852, Thermo Scientific), TRIPZshRhoB (V2THS_172671, Thermo Scientific) or TRIPZshRhoC (RHS4743, Thermo Scientific) vectors, 2 μg of pCMV-dR8.91 (Delta 8.9) plasmid and 1 μg of VSV-G, according to the manufacturer's protocol. The TRIPZ Inducible Lentiviral Empty Vector shRNA Control (RHS4750, Thermo Scientific) was used as a control lentiviral vector. The vectors above are engineered to be Tet-on and produce tightly regulated induction of shRNA expression in the presence of doxycycline (500 ng/ml). Additionally, in these vectors, turboRFP and shRNA are part of a single transcript, allowing the visual marking of shRNA-expressing cells. Forty-eight hours after the transfection, the supernatants were harvested, filtered and stored at -80°C. The supernatants were applied to U373MG and U373MG-IE1 cells in the presence of 8 μg/ml Polybrene (Sigma-Aldrich, USA). Transduced cells were selected after 48 hours using puromycin (1 μg/ml) (Sigma-Aldrich, USA) and maintained in the presence of this antibiotic.

Immunofluorescence Analysis

For immunofluorescence, 1×10^5 U373MG cells, transduced with the empty vector (EV) lentivirus vector and induced with doxycycline, were plated on glass coverslips in 24-well plates. The cells were subsequently infected with the recombinant HCMV AD169/IE2-EGFP virus expressing IE2 fused to the enhanced green fluorescent protein [57] at MOI =3 pfu/cell. The cells were fixed with formaldehyde (4% [vol/vol] in PBS containing 2% sucrose) and the nuclei were stained with DAPI. Fluorescent images of were acquired with an epifluorescent Leica DMIRE2 microscope, equipped with a Leica DFC300FX digital camera.

MTT assay

MTT assay was performed to determine the cell proliferation rate of both the parental U373MG and the derivative U373MG-IE1 cells after the knockdown of RhoA, RhoB or RhoC proteins, either in the presence of absence of HCMV infection. Briefly, 2×10^6 cells were transduced with either the shRNA Empty Vector or the TRIPZshRhoA or the TRIPZshRhoB or the TRIPZshRhoC lentiviruses, induced with doxycycline and subsequently were either infected with HCMV (MOI=3 pfu/cell) or mock infected to quantify their proliferation rate at 1 and 3 days after infection. In each cell type, the yellow tetrazolium MTT reagent (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (Sigma Aldrich, cat. no. R8755) was added and incubated for 4 hours at 37°C followed by the addition of 150 μl MTT solvent (dimethyl sulfoxide-DMSO) for 15 minutes and finally the measurement of the absorbance at 590 nm with a reference filter of 620 nm. All experiments were performed in triplicates.

In vitro wound healing assay

U373MG and U373MG-IE1 cells were transduced either with the shRNA Empty Vector or TRIPZshRhoA, TRIPZshRhoB or TRIPZshRhoC lentiviruses and were grown until high confluency in 6-well plates. Twenty-four hours after plating, the cells were either infected with HCMV (MOI=3 pfu/cell) or mock infected and the monolayer were wounded with a pipet tip after 48 h post infection. Cell debris was removed by washing two times with serum-free medium and monolayer maintained in medium for 72 hours with or without HCMV. The wound closure was monitored at 0, 3, 6, 9, 12, 24, 48 and 72 h.p.i, using an epifluorescent Leica

DMIRE2 microscope, equipped with a Leica DFC300FX digital camera. For each experimental point 8 fields photographed and the cells migrating inside 3 mm of wound were counted. Each experiment was carried out in triplicates and the cell motility was quantified by ImageJ 1.4.3.67 analysis (Launcher Symmetry Software).

Time Lapse Microscopy

In chambered coverglass units (Lab Tek, Thermo Scientific) 1×10^5 cells were seeded and infected at MOI 3 pfu/cell with HCMV AD169 virus. Forty-eight hours after infection, the cells were transferred in humidified chamber on the microscope stage with 5% CO_2 at 37°C. Still images from live cells were taken every 15 minutes for 5 hours using an epifluorescent Leica DMIRE2 microscope, equipped with a Leica DFC300FX digital camera. Images were exported as TIFF files and were processed by Photoshop.

Morphometry

For cell shape analysis cells were plated in two-well coverglass units chambers and bright field and fluorescent images of live cells were acquired with an epifluorescent Leica DMIRE2 microscope, equipped with a Leica DFC300FX digital camera. The total area was measured with Metamorph Cellprofiler Software (Genome Biology).

Statistical analysis

All data shown represent independent experiments carried out in triplicates. The measurements were compared by analysis of logged data (Graphpad Prism) and the significant differences were determined using a one-way ANOVA.

Results

Generation of glioblastoma cells with stable knockdown of RhoA, RhoB and RhoC

To investigate the role of RhoA, RhoB and RhoC in glioblastoma cells in the context of HCMV infection, we first established glioblastoma cells which were devoid of the above Rho GTPases. Parental U373MG cells and cells stably expressing the HCMV IE1 protein, named U373MG-IE1, were transduced with the doxyxycline-inducible TRIPZ lentiviral vectors expressing shRNA specifically targeting RhoA, RhoB or RhoC. A doxycycline-inducible TRIPZshRNA lentiviral empty vector (EV) was also used, serving as a negative shRNA control vector. All lentivirus tansduced cells were subsequently selected with puromycin. The efficiency of silencing for each Rho GTPase was determined by Western Blot in total cell extracts (Fig. 1). RhoA or RhoC depletion did not affect the expression of the other isoform however, RhoA and to a lesser extent RhoC silencing induced RhoB overexpression, which is in agreement with previous studies [58, 59]. On the contrary, RhoB knockdown did not affect RhoA or RhoC expression levels.

Cell morphology of RhoA, RhoB and RhoC knockdown glioblasoma cells in uninfected and HCMV infected cells

We next examined the morphological changes caused by the knockdown of RhoA, RhoB and RhoC in glioblastoma cells, in the absence or presence of HCMV. In non-infected U373MG cells, depletion of RhoA resulted in an elongated cell shape compared to the control cells (Fig. 2A). An analogous phenotype with long and thin protrusions was observed in RhoA-depleted U373MG-IE1 cells (Fig. 2B). Similarly, RhoC-knockdown cells led to elongated, mesenchymal-like cells (Fig. 2A and 2B), an observation which is consistent with the depletion of RhoC in other cell lines [60, 61]. Increased cell spread was visualized in both U373MG and U373MG-IE1 uninfected cells when RhoB was silenced (Fig. 2A and 2B).

In the context of HCMV infection, both U373MG and U373MG-IE1 RhoA and RhoC-depleted cells retained the basic feature observed in non-infected cells as described above, showing thin protrusions (Fig. 3A and 3B). Interestingly, in the HCMV infected RhoB-knockdown cells, an increased number of cellular projections were formed, a phenomenon which

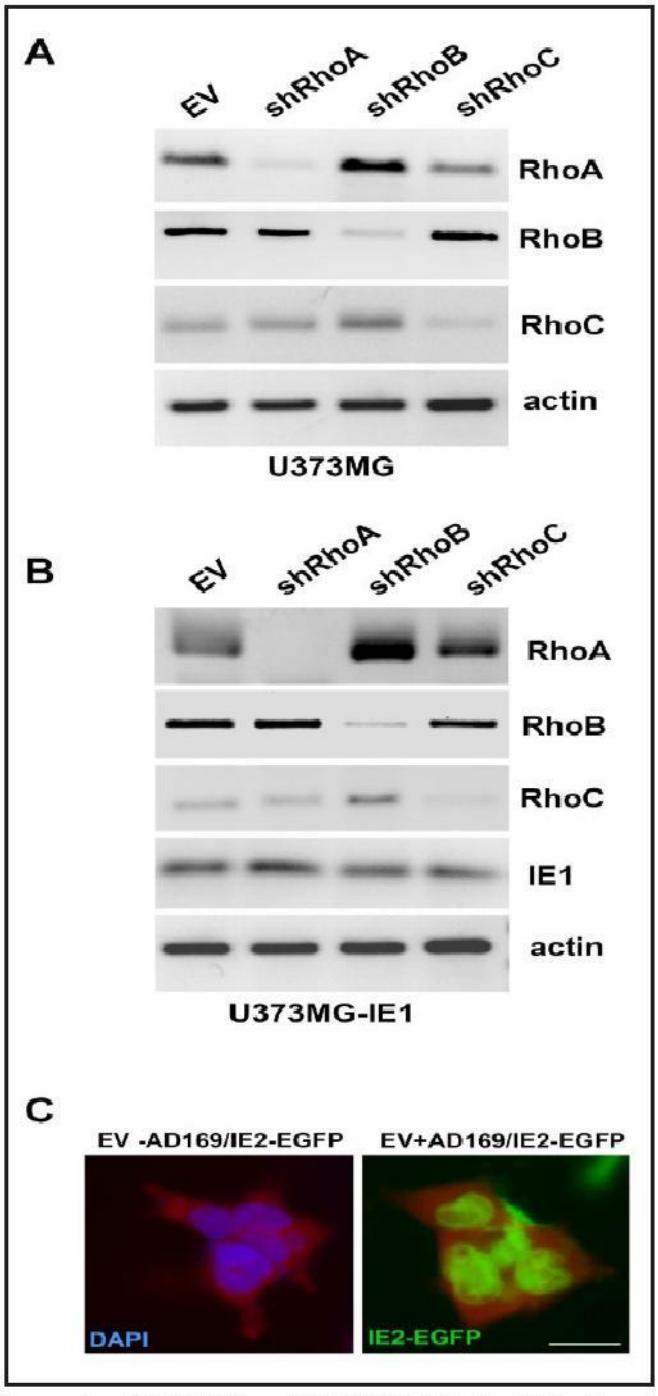

Fig. 1. Rho isoform specific knockdown in U373MG and U373MG-IE1 cells. The expression of RhoA, RhoB and RhoC were determined in (A) U373MG cells and (B) U373MG-IE1 cells by Western Blot from whole protein extracts derived from each cell line after transduction with the indicated TRIPZ shRNA lentivirus vectors, induction with doxycycline and selection with puromycin. Actin served as loading control.The stable expression of IE1 in U373MG-IE1 cells was also confirmed. (C) U373MG cells, transduced with the lentivirus empty vector and induced with doxyxyclynine, were infected with the recombinant HCMV AD169/IE2-EGFP virus at MOI=3 pfu/cell. Cells were fixed 8 hours after infection and nuclei were stained with DAPI. (bar: 10 μm).

has also been observed in primary HFF cells infected by HCMV [55]. Both HCMV infected glioblastoma cell lines with depleted each one of the three Rho isoforms were increased in volume, apparently due to the viral infection and the specific cytopathic effect HCMV causes.

The changes in the shape between the control cells and the three Rho proteins knockdown tested, both in uninfected and infected U373MG and U373MG-IE1 cells were measured using the Metamorph CellProfiler software. The analysis statistically further confirmed the alterations visualized by microscopy (Fig. 4).

Effect of HCMV on the proliferation rate of U373MG and U373MG-IE1 RhoA,RhoB and RhoC knockdown cells

We sought to determine the role of Rho GTPases in U373MG and U373MG-IE1 cells in uninfected and HCMV infected cells in terms of cell proliferation. Both cell lines were transduced with the appropriate shRNA lentivirus vectors knocking down RhoA, RhoB or RhoC, infected with HCMV AD169 when appropriate and subsequently tested in MTT assay. Depletion of either RhoB or RhoC in both the parental and the IE1 uninfected glioblastoma cells resulted in a significant inhibition of the proliferation rate compared to the control (empty vector) cells (Fig. 5A and 5B). Silencing of RhoA slowed down the cell growth of both parental and IE-1derivative uninfected cells compared to the control cells but to a

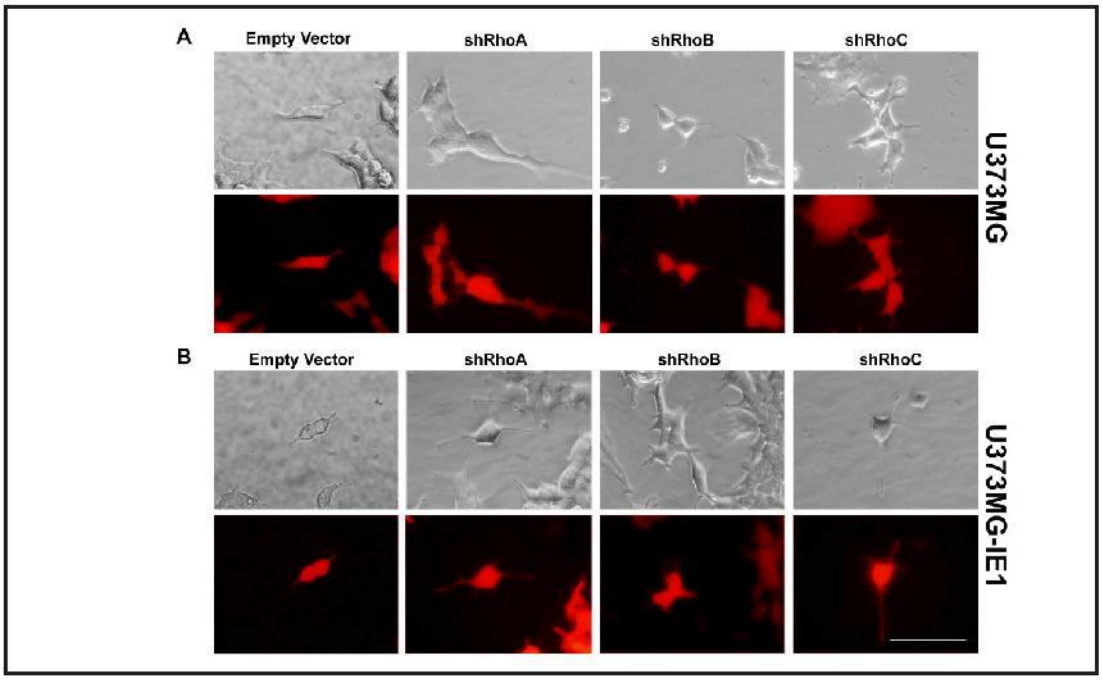

Fig. 2. Differential morphological changes in RhoA, RhoB and RhoC-depleted glioblastoma cells. U373MG and U373MG-IE1 cells were transduced with the indicated TRIPZ shRNA lentivirus vectors, induced with doxycycline and selected with puromycin. Doxycycline-treated TRIPZshRNA-expressing cells exhibit red fluorescence. Still images were obtained by timelapse microscopy 48h after plating (bar: 50 μm).

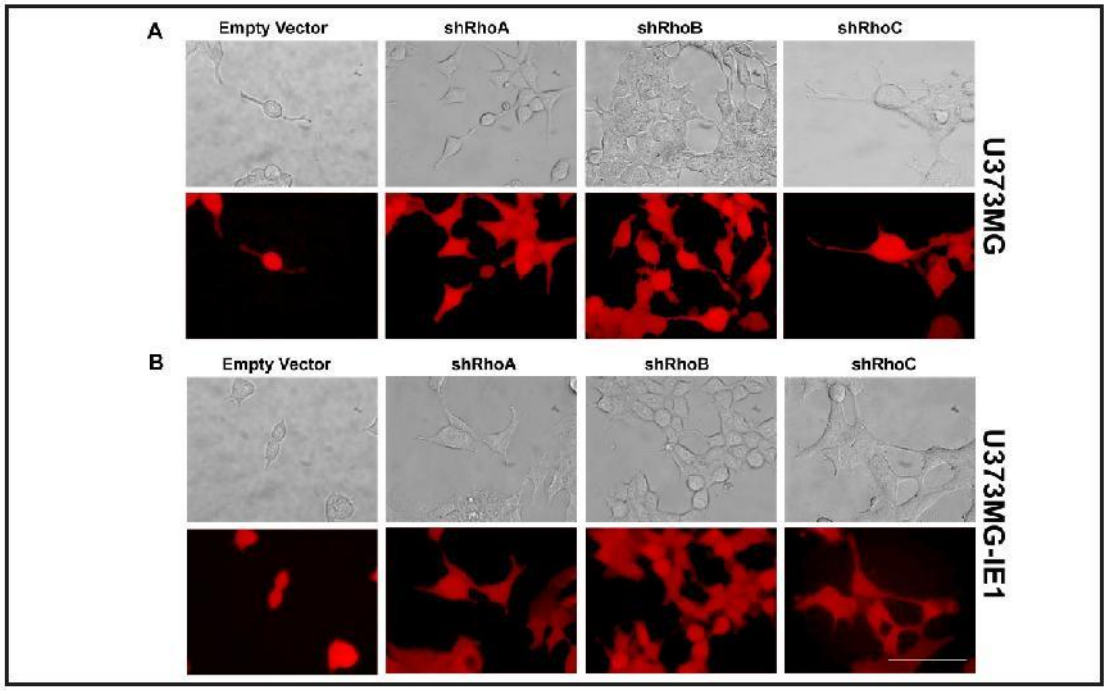

Fig. 3. Phenotype changes of RhoA, RhoB and RhoC-depleted HCMV infected glioblastoma cells. U373MG and U373MG-IE1 cells as in Fig. 2, were either mock infected or infected with HCMV AD169 (MOI=3 pfu/cell). Still images of live cells were captured 48h post infection (bar: 50 μm).

lesser extent compared to RhoB and RhoC silencing. HCMV infection alone did not affect the proliferation rate of either the U373MG or the U373MG-IE1 cells compared to the mock

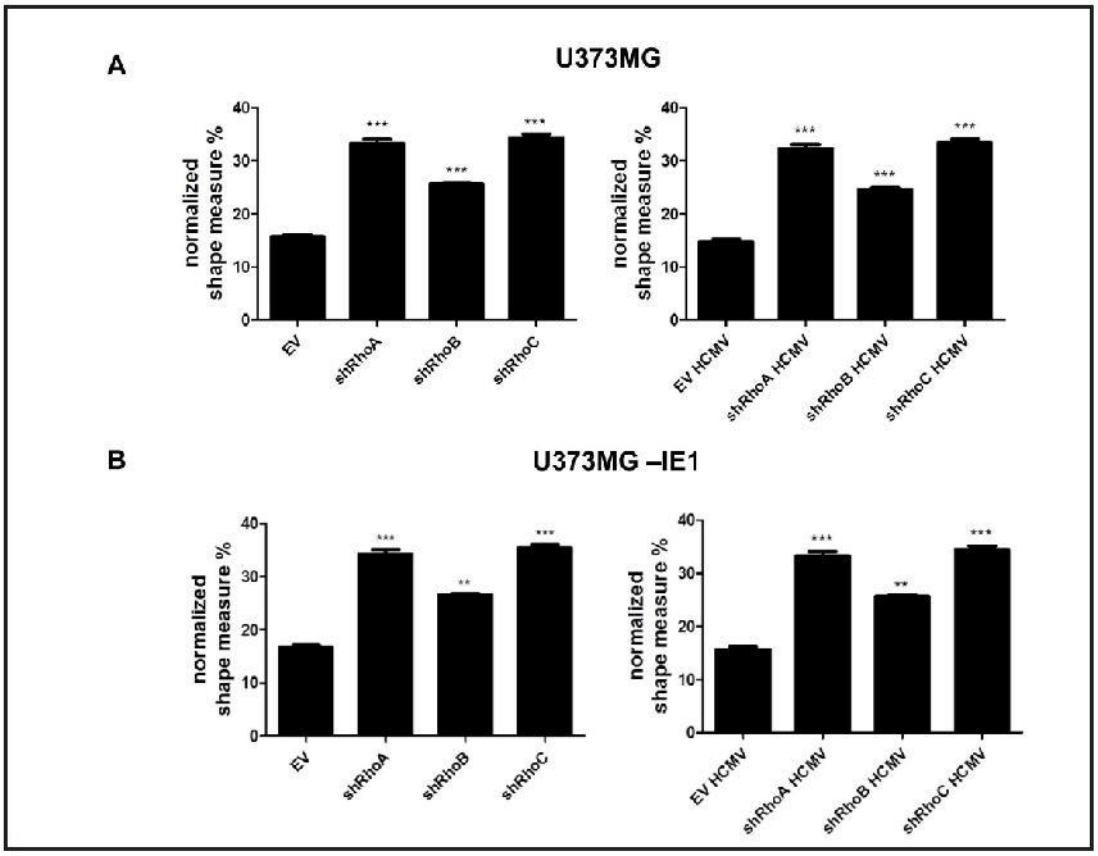

Fig. 4. Cell shape changes in RhoA, RhoB and RhoC knockdown uninfected and HCMV infected glioblastoma cells. U373MG and U373MG-IE1 cells were transduced with the appropriate TRIPZ shRNA lentivirus vectors, induced with doxycycline and selected with puromycin. HCMV AD169 at an MOI=3 pfu/cell was added in a subset of cells. Still images were captured 48 hours after infection, processed and analysed by Metamorph CellProfiler. The asterisks indicate statistical significance. ** indicate P = 0.001 to 0.01, *** indicate P = 0.0001 to 0.001. Data shown are means ± SD of three independent experiments.

infected cells. Interestingly, HCMV infection of U373MG RhoA knockdown and U373MG-IE1 RhoA depleted cells did not cause a statistically significant difference compared to the control cells ($p = 0.1385$), whereas the proliferation state of the HCMV infected of RhoB or RhoC knockdown cells was inhibited ($p = 0.0941$ and $p = 0.0089$ respectively) (Fig. 5A and 5B).

Depletion of RhoA, RhoB or RhoC inhibits random migration

To characterize the function of RhoA, RhoB and RhoC in cell motility, we initially tested the migration of the corresponding free-moving Rho depleted U373MG and U373MG-IE1 cells (Supplementary movies: http://www.med.uoc.gr/research-lab-clinical-virology.php). Cells transduced with the empty vector often showed a long and polarized morphology whereas RhoA, RhoB and RhoC knockdown cells exhibited narrow lamellipodial structures at each projection and small dynamic protrusions along the elongated sides. When analyzing the route of individual cells from each Rho depleted cell type during 300 min migration period by timelapse microscopy, we found that the cells displayed a much shorter translocation than the control cells. Remarkably, the movement of RhoA silenced cells was dramatically limited, lacking their parental polarization and rather presenting an amoeboid fashion with cycles of expansion and contraction of the cell body. Infection with HCMV of the same cells did not cause significant changes in their motility and they rather showed reduced random migration speed. On the contrary, HCMV infected U373MG-IE1 cells exhibited a higher

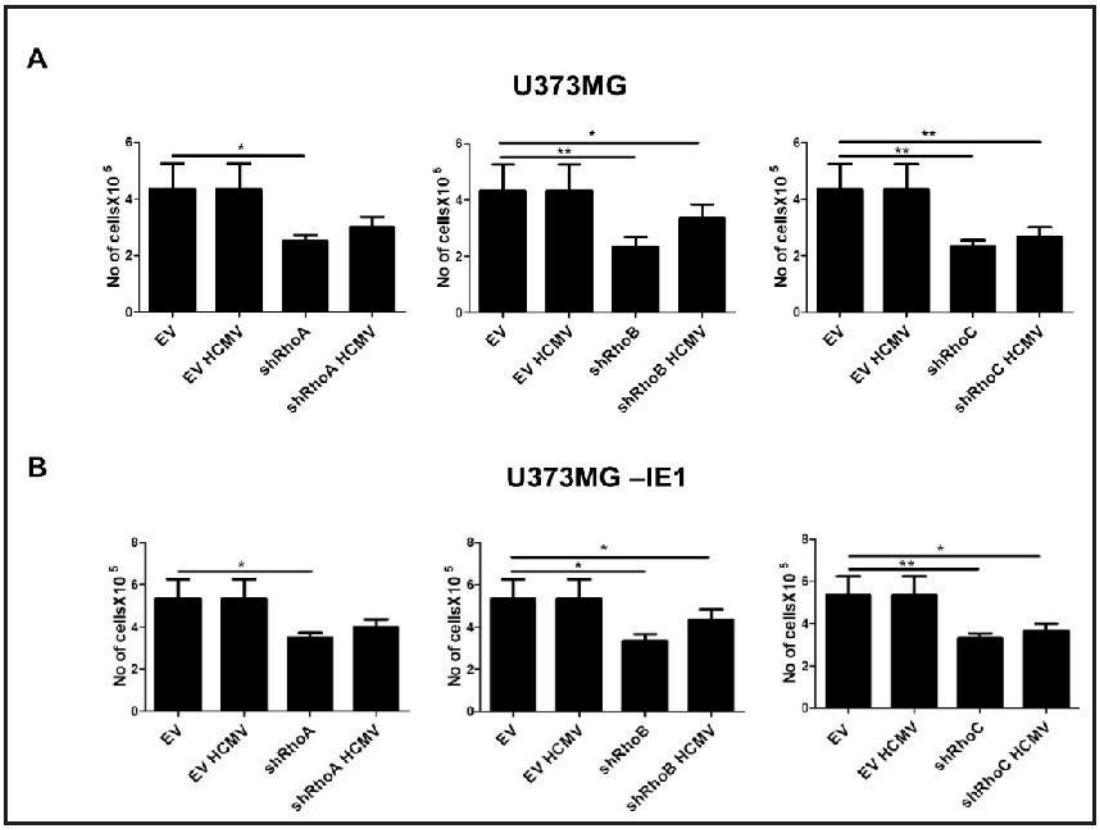

Fig. 5. Proliferation rate of RhoA, RhoB and RhoC knockdown uninfected and HCMV infected glioblastoma cells. The cells were transduced as in Fig. 2 and subsequently were either mock infected or infected with HCMV at MOI=3 pfu/cell and 3 days after infection the cell proliferation rate was measured employing MTT Assay. The asterisks indicate statistical significance. * indicate P = 0.01 to 0.1, ** indicate P = 0.001 to 0.01. Data shown are means ± SD.

motility speed, enhancing the defect in the migration caused by the knockdown of each Rho protein and showing longer paths. Even in this more favorable context for the IE1-expressing glioblastoma cells, the free-movement of shRhoA cells was the most severely impaired compared to the control cells.

Rho GTPases are required for glioblastoma cell migration

The role of RhoA, RhoB and RhoC in cell migration was explored in uninfected and HCMV infected parental U373MG and their derivative cells expressing the HCMV IE1 protein. Results showed that the knockdown of each of the three Rho isoforms significantly decreased the average migration rate of U373MG cells compared to empty vector cells, as determined by wound healing assays. Moreover a differential reduction in the speed of cell movement was monitored, depending on which Rho protein was depleted. Among all three Rho proteins tested, the healing rate was considerably faster in RhoC knockdown cells, moderately slower in RhoB depleted cells while strikingly, the movement of RhoA silenced cells was significantly limited and they appeared almost stuck throughout the course of wound healing assay (Fig. 6A). The aforementioned observations in Rho depleted cells did not change upon infection of the cells with HCMV (Fig. 6B). The migration rate of U373MG-IE1 cells was also decreased in RhoA, RhoB and RhoC knockdown cells compared to the empty vector cells. However, this difference was remarkable compared to the parental cells and apparently, the expression of IE1 protein accelerates cell migration compared to the non-IE1 expressing cells (DATA NOT SHOWN). In contrast to Rho depleted parental glioblastoma cells, HCMV infection of the U373MG-IE1 cells after knockdown of RhoA, RhoB or RhoC restored the migratory behavior

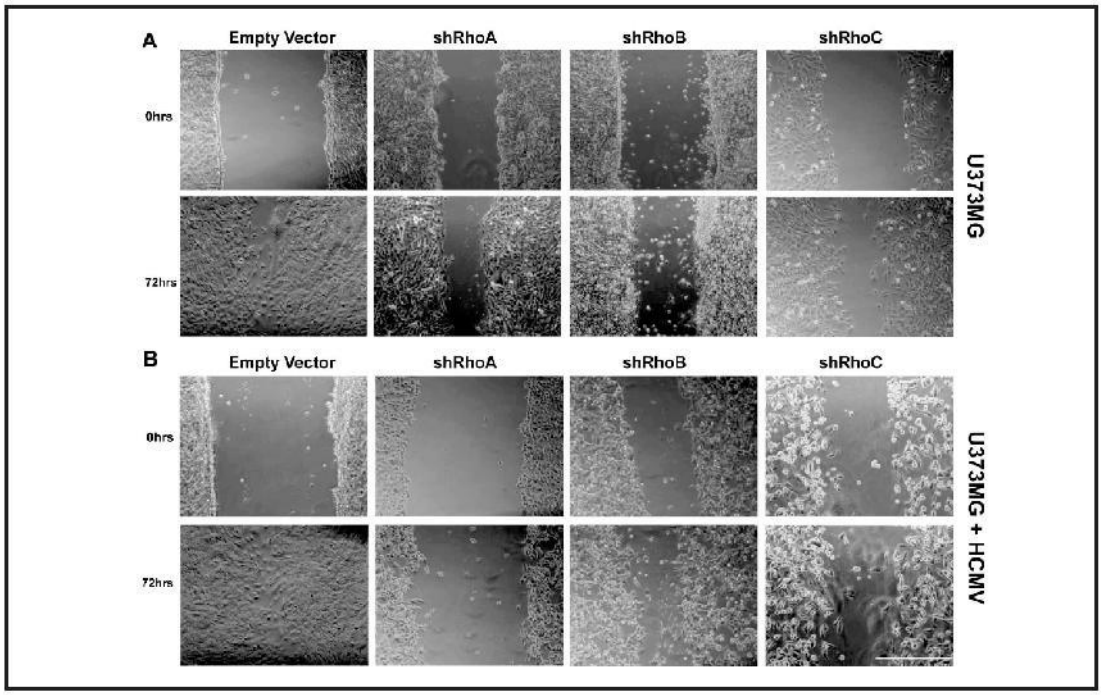

Fig. 6. Effect of RhoA, RhoB and RhoC depletion on wound healing migration in uninfected and HCMV infected U373MG cells. U373MG were plated in 6-well plates, treated as in Fig. 2 and were either HCMV infected (MOI=3 pfu/cell) or not. The monolayer was "wounded" 48 hours post infection with a 10 μl sterile pipette tip and the detached cells were removed. Images were taken at 0, 3, 6, 9, 12, 24, 48 and 72 h after the scratch to monitor cell motility by timelapse microcopy. The initial (0 h) and final (72 h) time points of the assay are presented. (bar: 50 μm).

of the cells at comparable levels to the empty vector cells, either in the presence or absence of viral infection.

The area of the wounds was also recorded at different time points (3, 6, 9, 12, 24, 48, 72 hours) following the generation of the wounds. A marked delay in wound closure was observed in RhoA, RhoB or RhoC knockdown U373MG cells compared to the empty vector cells (Fig. 7A). Calculations on the area of the wounds demonstrated that in control cells, only 53% ± 1.9% of the initial wound area was left as opposed to all three Rho depleted cells where the wounds areas did not to change significantly, as only 80% ±1.1% of them were covered. Close examination of cells at the wound edge revealed that control cells displayed a polarized phenotype, with cell protrusions perpendicular to the wound along with rapid elongation of cell projections. In contrast, cells with depleted Rho proteins exhibited a less evident polarized phenotype, with RhoA knockdown manifesting a more drastic disruption of polarity, followed by RhoB and RhoC silencing. Although these cells could still form protrusions at the wound edge, their directions were more random and a substantial proportion of cells displayed multiple short protrusions or protrusions parallel to the wound.

These phenotypes between the Rho depleted cells and the control cells remained unaltered during the course of HCMV infection. As regards the U373MG-IE1 cells, the defective closure capacity compared to the empty vector cells was also true when RhoA, RhoB or RhoC were silenced whereas HCMV infection of the same cells resulted in a remarkable increase in the closure efficiency of the wounds (Fig. 7B). These data collectively indicate that RhoA, RhoB and RhoC play a favorable role in cell migration of glioblastoma overexpressing IE1 protein cells and also that HCMV confers a migration advantage to these cells in Rho proteins depleted cells.

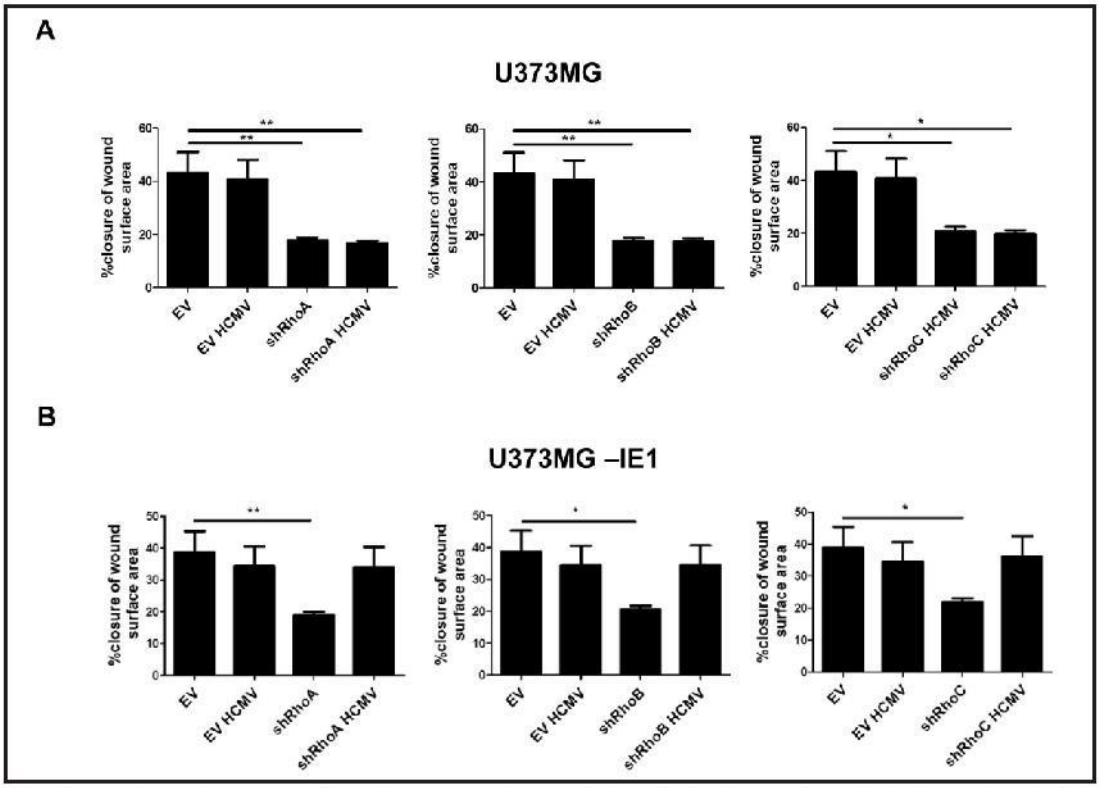

Fig. 7. Quantification of wound healing assay in RhoA, RhoB and RhoC knockdown U373MG and U373MG-IE1 cells. Motility was quantified by measuring the decrease in the denuded area at 0 hours and 48 hours and presented as the average decrease in the number of pixels with standard deviation in three independent experiments. The asterisks indicate statistical significance. * indicate P = 0.01 to 0.1, ** indicate P = 0.001 to 0.01. Data shown are means ± SD of experiments in triplicate.

Discussion

In the current study, we demonstrate, for the first time, an implication of Rho GTPases in cell morphology, proliferation and migration in human cytomegalovirus infected glioblastoma cells. Foremost, we observed that knockdown of RhoA resulted in an elongated cell shape with thin protrusions in non-infected and HCMV infected U373MG as well as in their derivative U373MG-IE1 cells. Likewise, depletion of RhoC led the cells to obtain an elongated, mesenchymal-like shape. The above observations are consistent with earlier studies depleting the same Rho isoforms both in prostate and breast cancer cells [59, 62]. The overexpression of HCMV IE1 protein alone did not bring about any additional effect regarding the phenotype of the Rho knockdown cells, thus excluding any direct modulatory role of IE1 on cell morphology. The morphological changes induced in the glioblastoma cells after RhoA and RhoC depletion remained evident, even in the context of HCMV infection, despite the cytopathic effect caused by the virus, highlighting an imposing role Rho GTPases on cell shape. Although Rho GTPases have been shown to enhance malignant transformation and proliferation rate, RhoB is rather assumed as a negative regulator of these processes [63, 64]. However, recent data have shown that RhoB expression is induced under conditions such as DNA damage, or treatment with growth factors or cytokines, suggesting that under particular circumstances, RhoB may favor towards human malignancy, including glioblastoma tumors [65, 66]. In the present study, we show that when RhoB is silenced, both parental and their derivative-IE1 cells show a reduction in spread area compared to the RhoA or RhoC knockdown cells, an effect which has attributed to subsequent reduction of total surface levels of b1 integrin [67, 68]. Moreover, in the presence of HCMV, an increased

number of cellular projections was formed, a phenomenon which has also been observed in HCMV infected primary HFF cells, particularly at the late stages of lytic infection [49]. These findings further corroborate the idea of a key role for RhoB in the regulation of cell shape.

Regarding to the proliferation rate of uninfected parental and IE1-expressing glioblastoma cells, the deficiency of RhoB or RhoC resulted in a significant inhibition of their proliferation state while RhoA deficiency reduced the cell growth of both U373MG and U373MG-IE1 cells to a lower degree. This difference reflects the evidence that RhoA regulates actin polymerization, cell adhesion and myosin activity [7, 69] whereas RhoB and RhoC control cell survival and cell proliferation, respectively [70]. This concept is furthermore supported by our observation that HCMV infection of U373MG and U373MG-IE1 RhoA depleted cells does not seem to affect significantly their proliferation rate, whereas the proliferation state of the HCMV infected of both parental and U373MG-IE1 RhoB or RhoC knockdown cells is statistically significantly inhibited. HCMV infection has been involved in human tumors influencing cell properties such as migration, invasion or cell signaling [31, 32, 71]. The results exploring the Rho GTPases silencing in association to cell proliferation of these cells and in the context of HCMV infection, indicate that RhoB and RhoC depletion in U373MG gliomablastoma cells rather results in the inhibition of the oncomodulatory effects of the virus. Interestingly, HCMV infection partially restored the growth rate of the RhoA knockdown glioblastoma cells, suggesting that the virus can differentially interfere with the proliferation state of the cells, depending on which Rho GTPase is depleted. This was also the case in the U373MG-IE1 RhoA knockdown cells. Apparently, the expression of IE1 alone and despite the oncogenic properties this protein may possess, it was not sufficient to confer a proliferation advantage to these cells and the active viral gene expression is required.

The alterations in the free movement of Rho knockdown cells were also monitored. The cells display diminished movement with U373MG and U373MG-IE1 depleted RhoA, RhoB or RhoC cells lacking their polarization and rather presenting an amoeboid formation with cycles of expansion and contraction of the cell body reinforcing the evidence that Rho GTPases contribute to different features of cancer, such as invasion and metastasis [72]. Infection with HCMV does not change significantly their motility and they rather show reduced random migration speed. In contrast, HCMV infected U373MG-IE1 cells exhibit a higher motility rate, enhancing the defect in the migration caused by the deficiency of each Rho protein. It is worth noting the fact that even in the absence or presence of HCMV, parental and their derivative IE1-knockdown RhoA cells exhibited the lowest motility rate even in this more favorable for the IE1-expressing glioblastoma cells context, providing an additional proof reinforcing the significant role of RhoA to promote cell migration [73, 74].

The aforementioned observations prompted us to investigate moreover the average migration of non-infected and infected U373MG depleted one of the three Rho isoforms cells, with RhoB and RhoC knockdown cells having the faster healing rate and in accordance with studies highlighting the role of these GTPases mainly in malignant transformation and to a less extend in motility [75], whereas the movement of RhoA silenced cells is decreased (Fig. 6A and 6B), revealing once more the differential role of RhoA, RhoB and RhoC in migration and invasion [76-78]. Interestingly, even if the migration rate of non-infected U373MG-IE1 depleted RhoA, RhoB and RhoC cells was also decreased, however, in the presence of HCMV, these cells partially restored their increased motility rate in migratory state, in contrast to the Rho depleted parental glioblastoma cells. This acceleration in cell migration could be due to the expression of viral genes, including IE1 protein since earlier studies have demonstrated that the IE1 protein suppresses apoptosis and facilitates oncogenesis [79, 80] and more recent studies that positively contributes to the replication cycle of HCMV [81, 82].

Taken together, our data suggest an important implication of RhoA, RhoB and RhoC GTPases in morphology, proliferation and motility of both uninfected and HCMV infected U373MG glioblastoma cells. HCMV has been shown to manage signaling networks in infected cells, including the activation of G proteins [83] in order to reserve infection and viral spread. In this study, we display that downregulation of RhoA, RhoB and RhoC proteins in infected glioblastoma cells decreases their proliferation rate and their migration status and inhibits

HCMV from worsening the malignant glioma pathogenesis. Interestingly, the presence of the HCMV Immediate-Early protein IE1 facilitates HCMV to retain its oncomodulatory advantage in these RhoA, RhoB and RhoC depleted cells [84]. Therefore, we suggest a possible direct or indirect involvement of Rho small GTPases during HCMV infection with potential role in human glioblastoma cells.

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Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interest and nothing to disclosure.

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