Insights into human adenovirus type 26-induced inflammasome activation in human epithelial cells: impact of clathrin-mediated endocytosis

Ana Runtić[1], Lucija Lulić Horvat[1], Davor Nestić[1,*], Isabela Drašković[1], Dragomira Majhen[1]

[1]Laboratory for Cell Biology and Signalling, Ruđer Bošković Institute, Division of molecular biology, Bijenička cesta 54, Zagreb, HR-10000, Croatia

*current address: Department of Biochemistry, University of Zürich, Zürich, Switzerland

INTRODUCTION

Human adenoviruses (HAdVs) are non-enveloped, double-stranded DNA viruses. As a low-seroprevalence type, human adenovirus type 26 (HAdV-D26) has been extensively investigated as a potential vector for vaccine development and gene therapy. Previous findings from our laboratory demonstrated that HAdV-D26 utilizes integrin αVβ3 for entry into epithelial cells (1). Furthermore, inhibition of clathrin-mediated endocytosis (CME) was shown to retain HAdV-D26 in the proximity to the cell membrane and impair its intracellular trafficking, while significantly enhancing viral internalization and infection efficiency in human epithelial cells (2). However, how HAdV-D26 interact with the innate immune system *in vitro* remains poorly understood. An important part of the inflammatory response is the inflammasome, a multiprotein complex that activates caspase-1 and promotes the maturation of the pro-inflammatory interleukins IL-18 and IL-1β. There is only one report regarding HAdV-D26 intracellular trafficking proposing that HAdV-D26 triggers innate immunity by causing TLR- and cathepsin-dependent inflammasome activation. Here we hypothesized that CME inhibition may modulate the cellular response to HAdV-D26 infection.

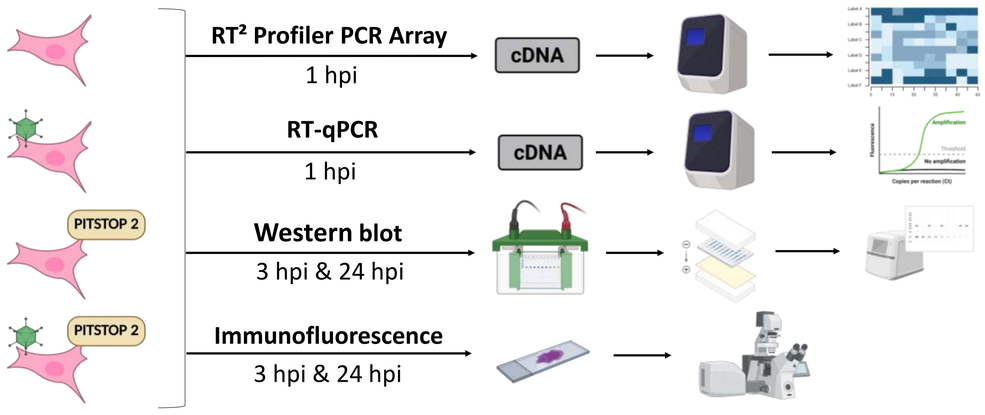
AIM

The aim of this study was to investigate the effect of CME inhibition on inflammasome activation in human epithelial cells infected with HAdV-D26.

METHODS

Cell lines: A549 (human lung carcinoma) and A549-E6 cell clone with increased expression of αVβ3 integrin

CME inhibition: Clathrin-mediated endocytosis was inhibited using 20 µM Pitstop 2 (Pts2)



hpi, hours post infection

RESULTS

INFLAMMASOME-RELATED GENE PROFILING

HAdV-D26 infection increases the expression of several inflammasome-related genes (e.g. NLRP3, AIM2, CARD18 and IFNG) in A549 and A549-E6 cells, both in the abscence and presence of CME inhibition.

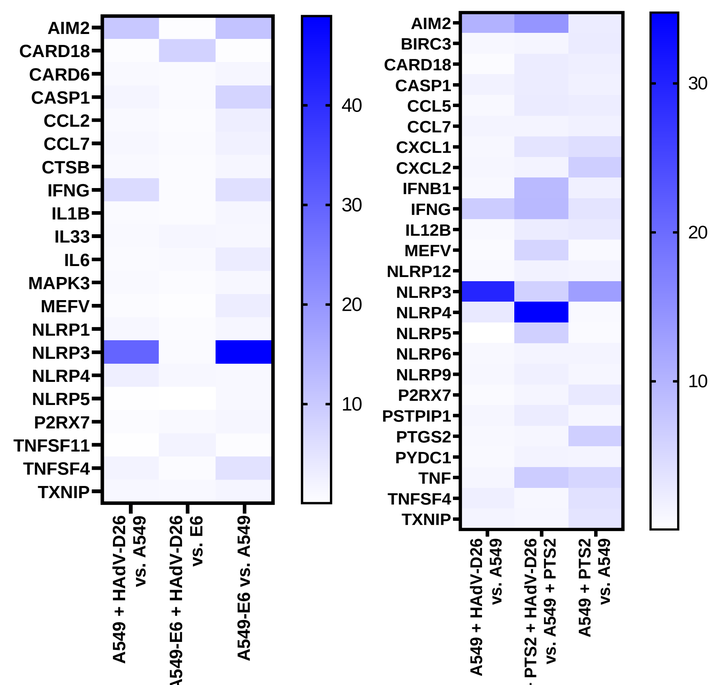


Figure 1. Gene expression profiling using the RT² Profiler PCR Array Human Inflammasomes in A549 and A549-E6 cells infected with HAdV-D26 and/or treated with Pts2. Heatmaps show the most differentially expressed inflammasome-related genes in A549 and A549-E6 cells following CME inhibition with Pts2 and HAdV-D26 infection for 1h at 10^4 pp/cell of virus.

TARGETED GENE EXPRESSION ANALYSIS

HAdV-D26 infection decreases *CASP1* and *IL18* expression and increases *IL1B* expression in A549 cells, while decreasing the expression of all three genes in A549-E6 cells. In contrast, HAdV-D26 infection and CME inhibition increase *CASP1, IL1B*, and *IL18* expression in both cell lines.

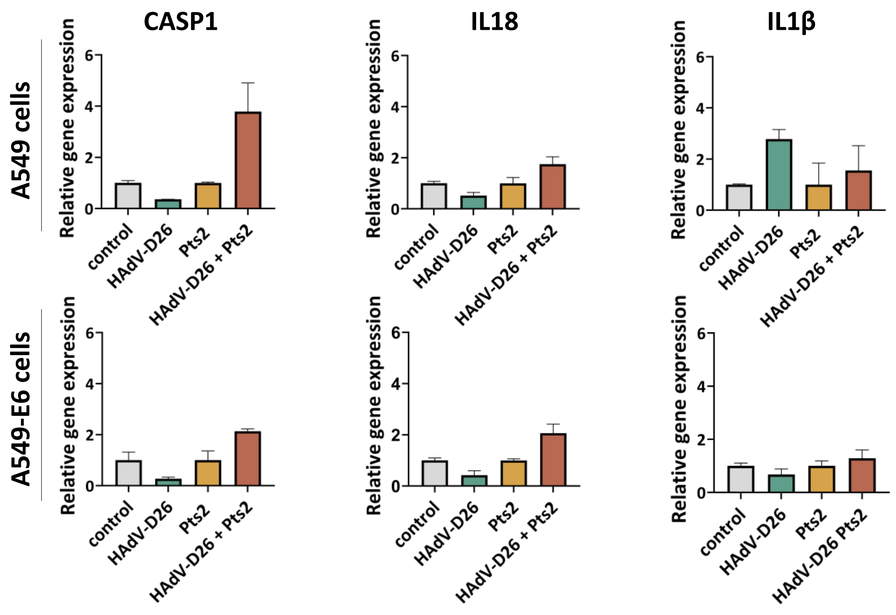


Figure 2. Relative gene expression of *CASP1*, *IL18* and *IL1B* in A549 and A549-E6 cells infected with HAdV-D26 and/or treated with Pts2. Cells were infected with HAdV-D26 for 1 h at 10^4 pp/cell. Gene expression was determined by RT-qPCR, normalized to *GAPDH* and the respective control (non-infected and non-treated cells or cells treated with Pts2), and is presented as mean ± SD.

INFLAMMASOME-RELATED PROTEIN EXPRESSION

Inflammasome-related protein levels in A549 and A549-E6 cells remain largely unchanged following HadV-D26 infection and CME inhibition at 3 and 24 hpi, with no detectable activation of caspase-1 and IL-18.

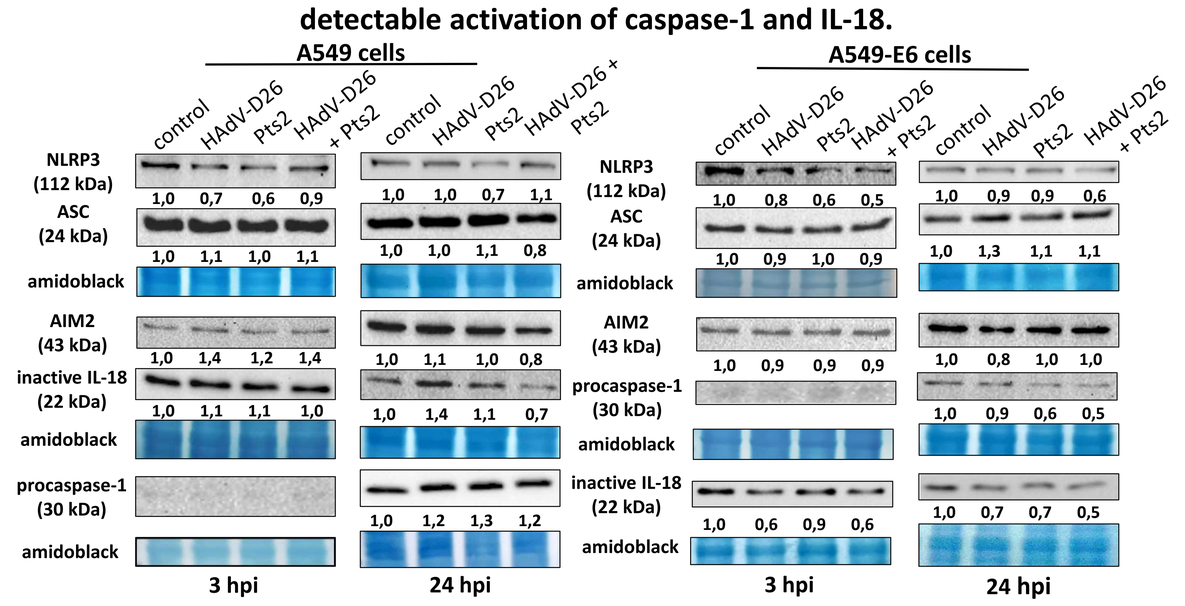
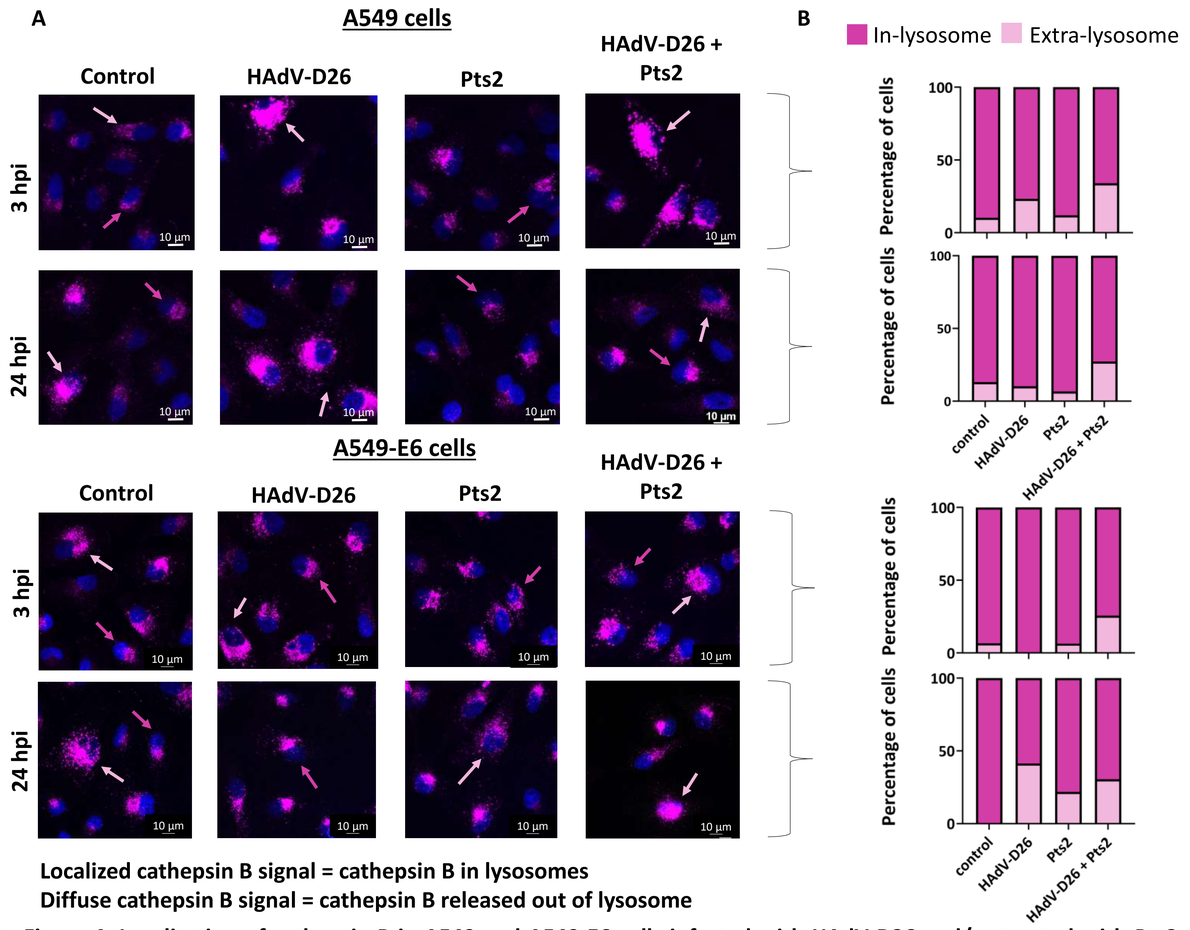


Figure 3. Relative expression of inflammasome-related proteins in A549 and A549-E6 cells infected with HAdV-D26 and/or treated with Pts2. Protein expression was analysed by Western blot at 3 hpi and 24 hpi with 10^4 pp/cell of HAdV-D26. Band intensities were quantified by densitometry and normalized to the corresponding total protein level stained with amido black. Presented values show protein expression relative to non-infected and non-treated cells (control). Amido black staining was used as a loading control.

CATHEPSIN B REDISTRIBUTION

HAdV-D26 infection promotes a more diffuse cathepsin B signal at 3hpi in A549 and 24hpi in A549-E6 cells, while combined infection and CME inhibition further increase the diffuse signal in both cell lines and at both time points.



Localized cathepsin B signal = cathepsin B in lysosomes
Diffuse cathepsin B signal = cathepsin B released out of lysosome

Figure 4. Localization of cathepsin B in A549 and A549-E6 cells infected with HAdV-D26 and/or treated with Pts2. Cells were infected with 5×10^4 pp/cell of HAdV-D26. Cathepsin B localization was analysed by immunofluorescence and visualized using a confocal microscope. A) Representative images show cathepsin B (magenta) and nuclei (blue). B) Graphs show the percentage of cells with in-lysosome and extra-lysosome cathepsin B signal. Light pink arrows indicate cathepsin B signal in extra-lysosomal space and dark pink ones indicate cathepsin B signal in lysosome.

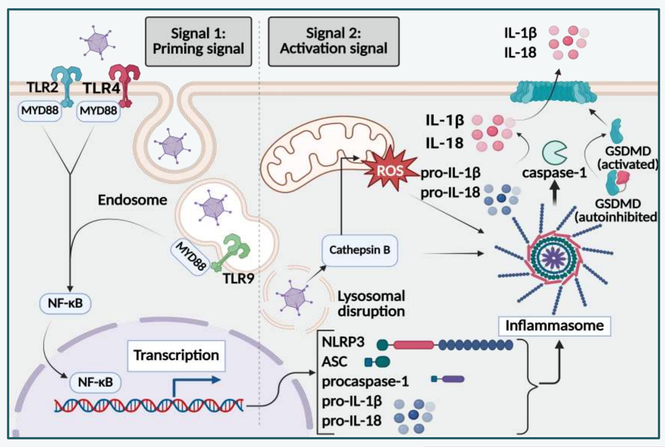


Figure 5. Schematic illustration of a potential NLRP3 inflammasome activation pathway following HAdV-D26 infection. Proposed scheme integrates the findings of this study with current knowledge of NLRP3 inflammasome activation. Our current working hypothesis highlights lysosomal disruption and cathepsin B release from lysosomes as a potential link between HAdV-D26 infection and NLRP3 inflammasome activation in human epithelial cells. The model was created with *BioRender.*

CONCLUSIONS

- CME inhibition increases *CASP1*, *IL1B* and *IL18* expression in A549 and A549-E6 cells infected with HAdV-D26 for 1 hour.
- No substantial changes in expression of NLRP3, AIM2 and ASC or activation of procaspase-1 and pro-IL-18 were observed in A549 and A549-E6 cells 3 and 24 hours post HAdV-D26 infection.
- HAdV-D26 infection promotes cathepsin B redistribution in A549 and A549-E6 cells 3 and 24 hpi, suggesting lysosomal disruption as a potential signal involved in inflammasome activation.
- Inflammasome assembly as well as other components of inflammasome remain to be investigated to assess inflammasome activation.

References: **(1)** Integrin αvβ3 Is Required for Efficient Infection of Epithelial Cells with Human Adenovirus Type 26 (2019) Nestic D, Taco G. U, Jiangtao M, Roy S, Vellinga J, H. Baker A, Custers J, Majhen D. *J. Virol*. 93 (1) **(2)** Human Adenovirus Type 26 Infection Mediated by αvβ3 Integrin Is Caveolin-1-Dependent (2022) Nestić D, Custers J, Švec D, Majhen D. *Microbiol Spectr.* e01097-22.