Differences in susceptibility of human and mouse macrophage cell lines to respiratory syncytial virus infection

1. Abstract

Objectives: Differences have been observed in the susceptibility of macrophage cell lines to Respiratory Syncytial virus (RSV) infection. In this study we evaluated if the type of macrophage cell-line and RSV strain used have an influence on infectivity and production of progeny virus. **Methods:** Both human and murine macrophage-like cell lines were infected with different RSV strains, both lab strains as well as clinical isolates. The infection was evaluated after 24h and 72h by immunofluorescence staining and microscopic analysis and the production of new virus particles was determined by plaque assay. **Results:** Susceptibility of macrophages for RSV was influenced by the RSV-strain used but is mostly depending on the macrophage cell line. Numbers of infected cells and virus production were generally very low or absent in murine cell lines. In human cell lines, clear infection was observed associated with production of new virus particles. **Conclusion:** Differences in susceptibility of macrophage cell lines for RSV infection are primarily related to the species of origin of the cell line but is also influenced by the RSV strain.

2. Introduction

The human respiratory syncytial virus (RSV; family Pneumoviridae; genus orthopneumovirus [1]) is the major cause of lower respiratory tract illness resulting in acute bronchiolitis and hospitalization of young children worldwide [2, 3]. Severe RSV infection is considered to be responsible for up to 200.000 deaths per year, but can also result in chronic respiratory problems, like recurrent wheezing and allergic asthma [4 - 6]. Although healthy non-elderly adults rarely require hospitalization, RSV is still considered the second important cause of medically significant respiratory tract disease [4]. Morbidity and mortality due to RSV is substantially increased in the elderly.

The immune system is an important factor, both in controlling RSV infection as well as for the development of disease, since both acute and chronic RSV pathology is characterized by a strong inflammatory immune response [2, 7]. Not only the adaptive immune response, but also innate immunity is important for both initial containment of the virus as well as the later immune responses [5, 8, 9]. Macrophages are the main resident innate immune cell in the broncho-alveolar space [7, 10-12]. Macrophages are a major source of cytokines, they play a role in the orchestration of inflammation and recruitment and activation of other immune cells [6, 8, 9, 13, 14]. Macrophages are thus key effector cells in the early response to an RSV infection and potentially for chronic pathology, but they may also potential target cells for RSV infection. RSV has a tropism for epithelial cells, but some studies suggest RSV can also infect macrophages [7, 8]. Since access to primary alveolar macrophages is limited, several studies were done on a variety of macrophage cell lines. Differences in the susceptibility of macrophage cell lines for RSV infection have been described however, ranging from productive infections [12, 14 - 17] to abortive infections, where the cells are infected and express RSV proteins but do not release new infectious virus particles [6, 18 - 20]. Other reports suggest that macrophage susceptibility for RSV infections depends on macrophage differentiation and maturation, claiming that monocytes are more susceptible for RSV than mature macrophages [12, 21]. The exact reasons for these differences are not clear but may be related either to differences in the RSV strains, or differences in the macrophage cell lines used. In this study, we compared the susceptibility of 5 different macrophage cell lines of both human and mouse origin, towards infection with different RSV strains. Our results show indeed major differences in susceptibility, mainly depending on the cells used, but also in part on the virus strain.

3. Materials and Methods

Cells and viruses

The MH-S murine alveolar macrophage cell line, the human monocytic cell lines U937 and THP-1 were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS). The murine macrophage-like cell lines RAW 264.7 and J774 and the human epidermoid carcinoma larynx cell line HEp-2 were maintained in DMEM containing 10% FCS. The RSV strains, A2, A1998/3-2, A2000/3-4, A2001/3-12 (BEI resources) and A2Line19F which expresses red fluorescent protein mKate2 upon infection [22] were grown on HEp-2 cell line. The RSV strain B1 was grown on Vero cells. For virus stock preparation, cells with a confluence of 80% were inoculated with RSV at a multiplicity of infection (MOI) of 0,5 in DMEM without serum for 2h. Afterwards the cells were maintained in DMEM containing 5% FCS at 37°C in a 5% CO2 atmosphere. Virus was collected after 2-3 days, when the first signs of cytopathology were observed. The supernatant was collected and centrifuged at 1000xg for 10 min at 4 °C. The virus suspension was aliquoted and stored at − 80 °C until use. For some experiments, RSV A2 virus was UV-inactivated using 200mJ/cm2 (CX-2000 UV crosslinker, UVP) and inactivation was confirmed by plaque assay on HEp-2 cells.

Infection

Macrophage cell lines and epithelial cell lines were seeded on glass coverslips at a concentration of 10^5 cells/ml and incubated overnight in 5% CO2 at 37°C. The human monocytic cell lines U937 and THP-1

were stimulated with respectively 25ng/ml and 10ng/ml PMA (phorbol myristate acetate) for 24h to promote attachment, and afterwards maintained in RPMI containing 10% FCS for 16h. Cells were inoculated for 2h with RSV at a MOI 1 - 100, in DMEM or RPMI depending on the cell type. Afterwards the cells were washed 2 times, fresh medium was added, and cells were incubated with RPMI or DMEM containing 10% FCS for 24h and 72h in 5% CO2 at 37°C. Finally, the supernatant and 2 wash fractions were pooled, snap frozen and stored at -80°C. The cells were fixed with 4% paraformaldehyde.

Immunofluorescence staining

Expression of RSV proteins in infected cells was detected through indirect immunostaining. To enable intracellular staining, cells were treated for 10 min at RT with 0,5% Triton X-100. Afterwards, the total viral antigens were stained with a goat polyclonal anti-RSV serum (Virostat) followed by a secondary AF488 or AF555 conjugated donkey anti-goat antibody (Life technologies). For some experiments, cells were stained with monoclonal human anti-F antibody palivizumab, leftovers were provided by the Department Pediatrics of the Antwerp University Hospital (S. Verhulst), with an AF488 conjugated secondary goat anti-human antibody prior to permeabilization to detect cell surface expression of the RSV F protein (Life technologies). Images were obtained using an Axio Observer inverted microscope and a Compact Light Source HXP 120C with Filter set 49, 10 and 20 for blue, green and red fluorophores respectively (Zeiss).

Plaque assay

The number of infectious virus particles in the supernatant was determined using a plaque assay on HEp-2 cells, as described [23, 24]. Cells were seeded in a 96-well plate at a density of $2x10^5$ cells/ml. After overnight incubation at 37°C in a 5% CO2 atmosphere, dilution series of the supernatant samples in DMEM were added to the cells. After 2h, the inoculum was removed and an overlay consisting of 0,6% Avicel (FMC Biopolymer, Brussel, Belgium) in DMEM containing 10% FCS was added. After an incubation of 3 days in 5% CO2 at 37°C the cells were fixed with 4% paraformaldehyde and permeabilized with 0,5% Triton X-100. The plaques were visualized through immunostaining with a goat polyclonal anti-RSV serum (Virostat), followed by a secondary HRP conjugated donkey anti-goat antibody (Life Technologies), followed by the HRP-substrate 1-step chloronaphtol substrate solution (Thermo Fisher), which results in a blue precipitation.

Statistical analysis

Every experiment was performed with three independent repeats, for which the average and standard deviation was calculated. The results were analyzed through a Student's t-test and p-values are considered significant if below 0,05. In the figures, significance is indicated with * if $p<0,05$ and ** if $p<0,01$. For each individual experiment where the analysis consists of cell counting on microscopic slides, counting was done in 5 different microscope fields, resulting in an average of 500 cells counted per experiment.

4. Results

Infection of different macrophage cell lines with different RSV strains

Different macrophage cell lines, and HEp-2 cells as a reference were inoculated with different RSV strains at a MOI 1, after which the cells were incubated for 24h at 37°C. The cells were fixed, permeabilized and stained, and the percentage of infected cells was determined by counting fluorescent cells. Clear differences were observed in the number of infected cells (Figure 1). Of the mouse cell lines, the J774 cells showed almost no infected cells, while for the MH-S and RAW 264.7 cells a low percentage of RSV infected cells was observed (1,4% to 10%). Both human cell lines, THP-1 and U937, were susceptible for RSV infection with 4% and 30% infected cells respectively. The RSV

strain had a drastic effect on the percentage of infected cells for human cells, while for mouse macrophages, only minor differences were observed. The reference A2 strain always resulted in a higher number of infected cells compared to the reference B1 strain. Both the cell line as well as the RSV strain used affected susceptibility, yet the species-origin of the cell type used seems to have the largest effect.

Since macrophages may stain intensely for viral antigen because of uptake of virus particles without infection, cells were also inoculated with UV-inactivated A2 RSV particles as a control. UV-treatment inhibits replication of the virus particles by genome degradation but leaves the virus particles intact [25, 26]. No staining of the cytoplasm was observed with the UV-inactivated particles (Figure 1), suggesting that the observed staining in cells inoculated with non-inactivated virus results from viral replication, and translation of new viral proteins and is not the result of uptake of viral. Furthermore, the recombinant RSV strain A2Line19, which expresses the red fluorescent protein mKate2 upon infection and virus replication was used to infect the cells. Analysis of the cells showed co-localization of cells stained green for viral antigens and red for mKate2 expression (Figure 2), further confirming that the cytoplasmic staining of viral antigens is indeed the result of production of new viral proteins.

Evaluation of infection at different MOI

The infection at MOI 1 resulted in most cell lines in a small percentage of infected cells, which could be the result of a low number of cells being actually permissive for RSV or because of a general lower susceptibility of the cells that could be overcome by using a higher concentration of virus in the inoculum. Therefore, cells were infected with RSV A2, B1 and one clinical isolate, A2000, at a MOI of 1, 3 or 10. Different results were obtained with the different cell lines. MH-S (murine), U937 (human) and the control HEp-2 showed a higher number of infected cells at MOI 10 (Figure 3). In contrast, the percentage infected cells with RAW 264.7 (murine) and THP-1 (human) cells declined with the increasing MOI, while infection remained very low for the cell line J774 at all MOI tested.

Kinetics of virus infection

To evaluate if RSV infection of the macrophage cell lines was productive or abortive, the number of infected cells and the production of new infectious virus in the supernatant at 24h and 72h p.i. were evaluated. Similarly, to the previous experiment, no RSV infected cells and no virus production was observed in the murine cell line J774. A limited number of infected cells was observed in RAW 264.7 and MH-S cells at 24h p.i., but at 72h p.i. the number of infected cells was even lower (Figure 4). In alignment with these findings, virus production was very low in both cell types at 24h p.i. and declined further at 72h p.i., except for the RSV A2 infected MH-S cells (Figure 5). In contrast, infected cells were clearly present in human macrophage cells at 24h p.i. Especially for the U937 cells, a high number of infected cells was observed that was similar as the percentage of infected cells at 24h p.i. in Hep-2 cells. Furthermore, the percentage of RSV-infected human macrophage cells at 72h p.i. was either similar or higher than the percentage of infected cells at 24h p.i., suggesting a productive infection. This was further confirmed by the detection of infectious virus in the cell culture supernatant at 24h p.i. At 72h p.i., a similar or higher amount of virus was detected in the supernatant, further indicating that virus infection in these cells results in production of new virus particles. To investigate if the sometimes-low amount of virus detected in the supernatant was not merely residual virus from the inoculum, the cell supernatant was collected 1 hour after replacing the inoculation-medium and infectious virus was quantified. For each RSV strain and macrophage line, this residual RSV concentration was comparable and ± $2{,}5x10^2$ PFU/ml.

Evaluation of surface expression of viral proteins

Clearly, not all macrophages are susceptible to RSV infection. J774 cells appear resistant to RSV infection, while RAW 264.7 are infected, but do not seem to produce infectious virus (abortive infection). A possible explanation for the minimal release of infectious particles, despite the obvious

expression of viral proteins, is the absence of expression of the viral envelope proteins on the cell membrane, which is vital in the assembly and release of RSV particles [3, 27, 28]. Therefore, the cells were infected with RSV and stained for surface expression of F with a monoclonal antibody and total expression of RSV-proteins with a polyclonal serum. Surface expression of the F-protein was clearly different between the cell types tested, ranging from 16,00% for an A2 infection in MH-S to 79,75% for A1998/3-2 infections in THP-1 (Figure 6), but these differences cannot explain the differences observed in the virus production.

5. Discussion/Conclusion

In this study, macrophage cell lines both from human and murine origin, which were used in previous studies with RSV and other viruses, were inoculated with different RSV strains and infection and virus production was evaluated. Different cell lines have different susceptibilities for a given RSV strain and susceptibility also differs within the same cell line for different RSV strains. The human cell lines were however clearly more susceptible for RSV and produced more virus particles. Since the human cell lines tested were treated with PMA prior to infection, this could have caused this difference. PMA stimulates and differentiates macrophages and several studies demonstrated that this can alter the susceptibility towards virus infections. PMA treatment increases infection of Ebola virus, but decreases the infection of Dengue virus [29 - 31]. Therefore, we also infected murine macrophage cell lines that were treated with PMA, but no change in susceptibility for the RSV strains was observed (results not shown). The lower susceptibility and the stronger restriction on the production of new virus particles compared to macrophage cell lines with human origin is possibly species related. Experiments with bone marrow-derived macrophages and alveolar macrophages of Balb\c mice and human blood monocyte-derived macrophages, infected with RSV A2 and A2Line19 confirm this lower susceptibility of macrophages of murine origin (data not shown). The effect of the RSV strain was more limited, with the exception of the B1 strain which showed lower infection levels in all cell lines. A possible explanation for this lower infection is that the RSV B1 was grown on Vero cells, which is common for most RSV B viruses as they grow better on these cells [32-34]. Yet the growth of RSV in Vero cells can have an effect on the attachment protein G, which may explain the observed lower infectivity in macrophages in this study [35].

Since infection at MOI 1 resulted in most cell lines in a small percentage of infected cells, it was questioned if this was due to only a small fraction of the cells being actually permissive for RSV. Therefore, the cell lines were infected with increasing MOI, the resulted infection differed between the cell lines. There were cell lines with an increased degree of infection, MH-S and U937, showing that the limited percentage of infected cells was not the result of a limited number of cells being permissive for RSV. Surprisingly the percentage of infection declined in other cell lines, RAW 264.7 and THP-1. Altered activation of the immune response to the different MOI's can be a possible explanation. This is previously described in case of a SeV infection of U937 where the infection with MOI 0,05 triggered a different IFN-I response than infection with MOI 5. In this case the profile of the expressed IFN-I subtypes was more varied with a low MOI, than during the infection with a higher MOI [36]. Since the immune response isn't solely dependent of virus, it is possible that RSV induces a different immune response depending on the cell line and the MOI.

All tested macrophage cell lines, except for the J774 cell line, were susceptible for RSV infection and allowed expression of newly synthesized viral proteins. Furthermore, infectious virus was detected in the supernatants of some cell lines, suggesting that new virus particles were produced. Low amount of virus was however detected for some cells, which may be residual virus of the inoculum. Therefore, the amount of residual virus in the supernatant was determined 1h after replacing the inoculum. The amount of residual virus was $\pm 2{,}5 \times 10^2$ PFU/ml, similar to the amount of virus detected in the supernatants of the infected cells. However, because infectivity of free RSV rapidly declines at 37°C and was shown to be abolished after 24h [19], it is plausible that the infectious virus detected after 24h and certainly after 72h is the result of the production of new viruses, albeit at very low levels. For

murine macrophage cell lines however, RSV infection was apparently abortive. The abortive character of RSV infection in macrophages was already observed and reported in different macrophage types, but the reason for this is not yet discovered [6, 18 - 20, 37]. A possible explanation for the observe abortive infections is the absence of envelope proteins on the cell surface. The presence of envelope proteins is necessary for initializing the formation and release of new particles [3 - 5, 27, 28]. In our study, human cell lines had a greater fraction of infected cells with surface expression compared the murine cell lines, but there was no apparent relation between surface expression of viral proteins and the production of new virus particles.

Type I interferons may also limit virus replication and production and release of viruses. Although all cell types can theoretically produce this cytokine, macrophages appear to be a major source of this cytokine, and therefore infection may be more restricted in these cells [10, 13, 18]. The effect that IFN-I specifically has on RSV infections was indicated by experiments with recombinant RSV strains, deficient in the non-structural (NS) proteins. Replication of NS-deficient RSV strains was poorly in IFN-I proficient cell lines such as A549, and this decrease in replication was not observed in IFN deficient cell lines, such as Vero cells [38]. However, Makris et al. recently showed that the abortive nature of RSV infection in primary murine macrophages was IFN-I independent. RSV infection in IFN-I deficient macrophages showed stronger anti-RSV staining in the cytoplasm and showed, in general, higher viral protein expression in contrast to wild type macrophages, but the infection remained abortive [6]. Type I interferons can therefore play an important role in limiting the infection of macrophages but is most likely not the determining factor whether the RSV infection is abortive or not.

This study clearly demonstrates that there are clear differences in infection between different macrophage-like cell lines and RSV strains. The differences are both related to the RSV strain and the cell line and are detected by both a difference in susceptibility as the degree of limitation in the release of new virus particles.

8. Statements

8.1. Acknowledgements

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8.2. Statement of Ethics

Ethical approval was not required since this work was performed using established cell lines and virus strains.

8.3. Disclosure Statement

The authors have no conflicts of interest to declare.

8.4. Funding Sources

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8.5. Author Contributions

A.H. and P.D. conceived and designed the experiments, analyzed the data and wrote the paper. A.H., A.L. performed the experiments. W.V.d.G., M.D.S., P.C. proofread the paper.

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10. Figure Legends

Figure 1: Infection of murine and human macrophage cell-lines with different RSV strains. Cells were inoculated with different RSV strains and 24h p.i., the cells were fixed, permeabilized and stained with goat polyclonal anti-RSV antibody and an AF488-labeled secondary donkey anti-goat antibody. The cells were also inoculated with UV-inactivated A2 RSV sample as negative non-infectious control. The percentage of infected cells was determined through counting the positively stained cells and the DAPI-stained cell nucleus. The results were analyzed with a Student's t-test and significance of $p<0,05$ (*) and $p<0,01$ (**) is indicated on the graph.

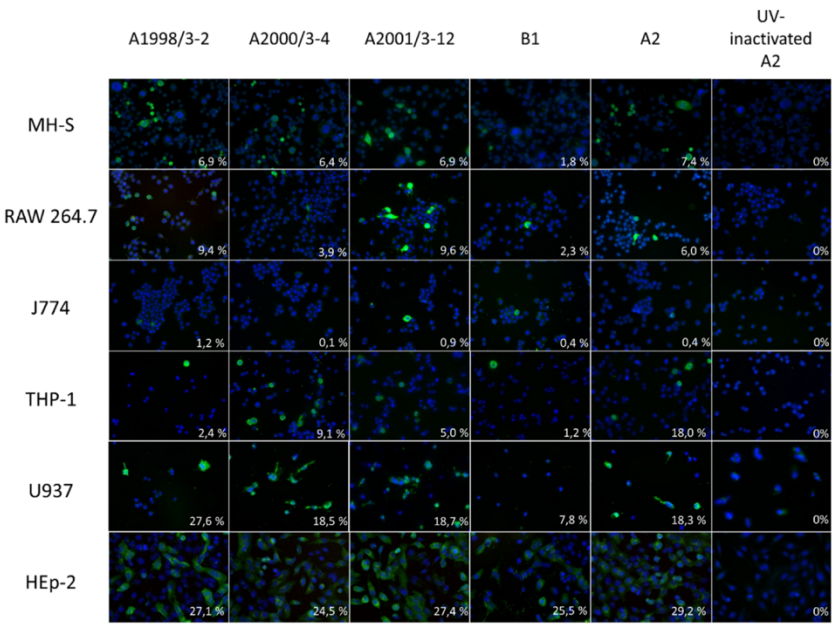
Figure 2: Demonstration of active transcription and translation of the viral genome. The cell lines which showed susceptibility for RSV infection were inoculated with the recombinant RSV strain A2Line19. After 24h, these cells were fixed, permeabilized and indirect stained with goat polyclonal anti-RSV antibody and an AF488-labeled donkey anti-goat antibody. The table shows the overlap of the staining patterns (first column), the expression of red fluorescence protein mKate2 by cells infected with A2Line19 RSV strain (second column) and the staining pattern resulted from the indirect staining of RSV antigens (last column).

Figure 3: Infection of murine and human macrophage cell-lines with RSV strains at different MOI. Cells were inoculated with RSV strains at MOI 1, 3 or 10 and 24h p.i., the cells were fixed, permeabilized and stained with goat polyclonal anti-RSV antibody and an AF488-labeled secondary donkey anti-goat antibody. The % infected cells was determined through counting the positively stained cells and the dapi-stained cell nucleus. The results were analyzed with a Student's t-test and significance of $p<0,05$ (*) and $p<0,01$ (**) is indicated on the graph.

Figure 4: Percentage of infection over time of the different macrophage cell lines for the different RSV strains. After inoculation, the cells were incubated for 24h or 72h. Subsequently, the cells were fixed, permeabilized and indirect stained with goat polyclonal anti-RSV antibody and an AF488-labeled donkey anti-goat antibody. The percentage of infected cells was microscopically determined for the two different time points. The results were analyzed with a Student's t-test and significance of $p<0,05$ (*) and $p<0,01$ (**) is indicated on the graph.

Figure 5: Determination of virus production in the supernatant of RSV infected cells. Supernatants were collected at 24h and 72h post inoculation, and the amount of infectious virus was determined by plaque assay on HEp-2 cells. The results were analyzed with a Student's t-test and significance of $p<0,05$ (*) and $p<0,01$ (**) is indicated on the graph.The dotted line represents the concentration of the residual virus in the supernatant immediately after inoculation.

Figure 6: Analysis of F protein surface expression in infected cells. The susceptible cell lines were infected with the different RSV strains for 24h. The presence of the viral F-protein on the cell surface was visualized by double staining. Prior to permeabilization, the cells were stained with monoclonal anti-F antibodies and an AF488-labeled secondary antibody, afterward the cells were permeabilized and stained with polyclonal anti-RSV serum and an AF555 labeled secondary antibody. The percentage of infected cells with surface expression was determined.


A1998/3-2
A2000/3-4
A2001/3-12
B1
A2
UV-inactivated A2
MH-S
RAW 264.7
J774
THP-1
U937
HEp-2
6,9 %
6,4 %
6,9 %
1,8 %
7,4 %
0%
9,4 %
3,9 %
9,6 %
2,3 %
6,0 %
0%
1,2 %
0,1 %
0,9 %
0,4 %
0,4 %
0%
2,4 %
9,1 %
5,0 %
1,2 %
18,0 %
0%
27,6 %
18,5 %
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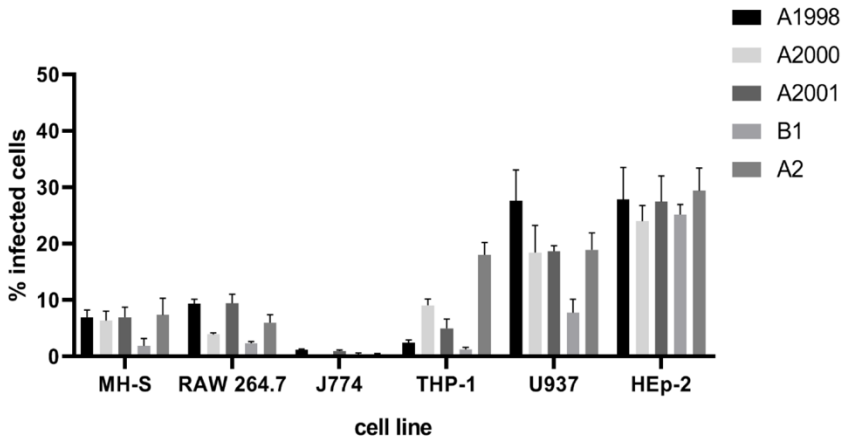
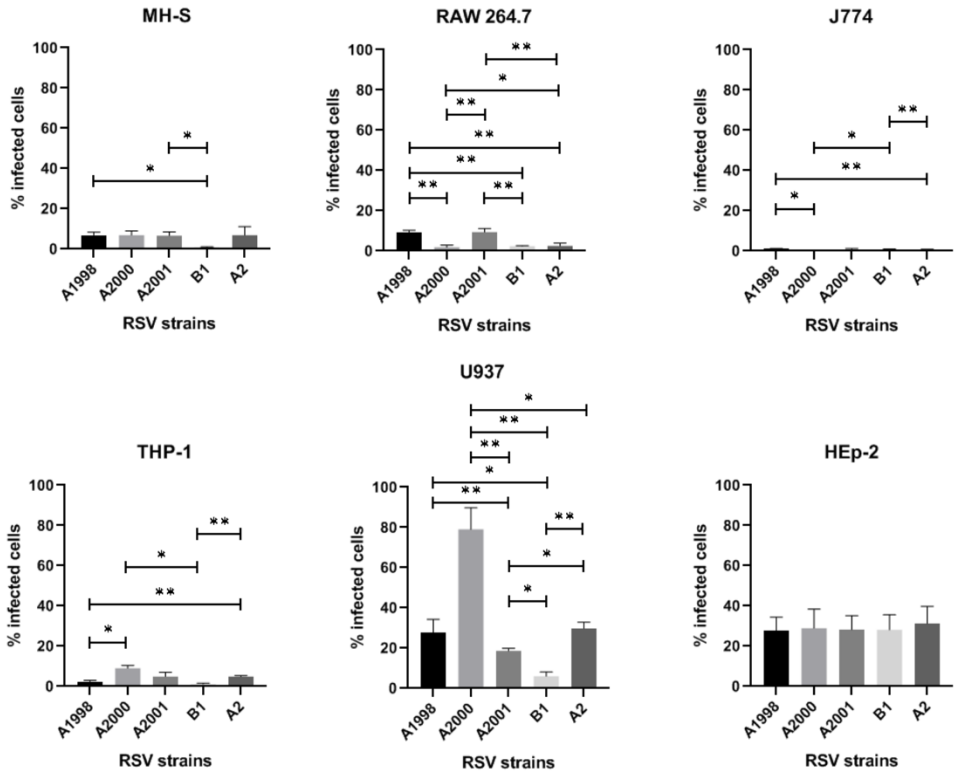
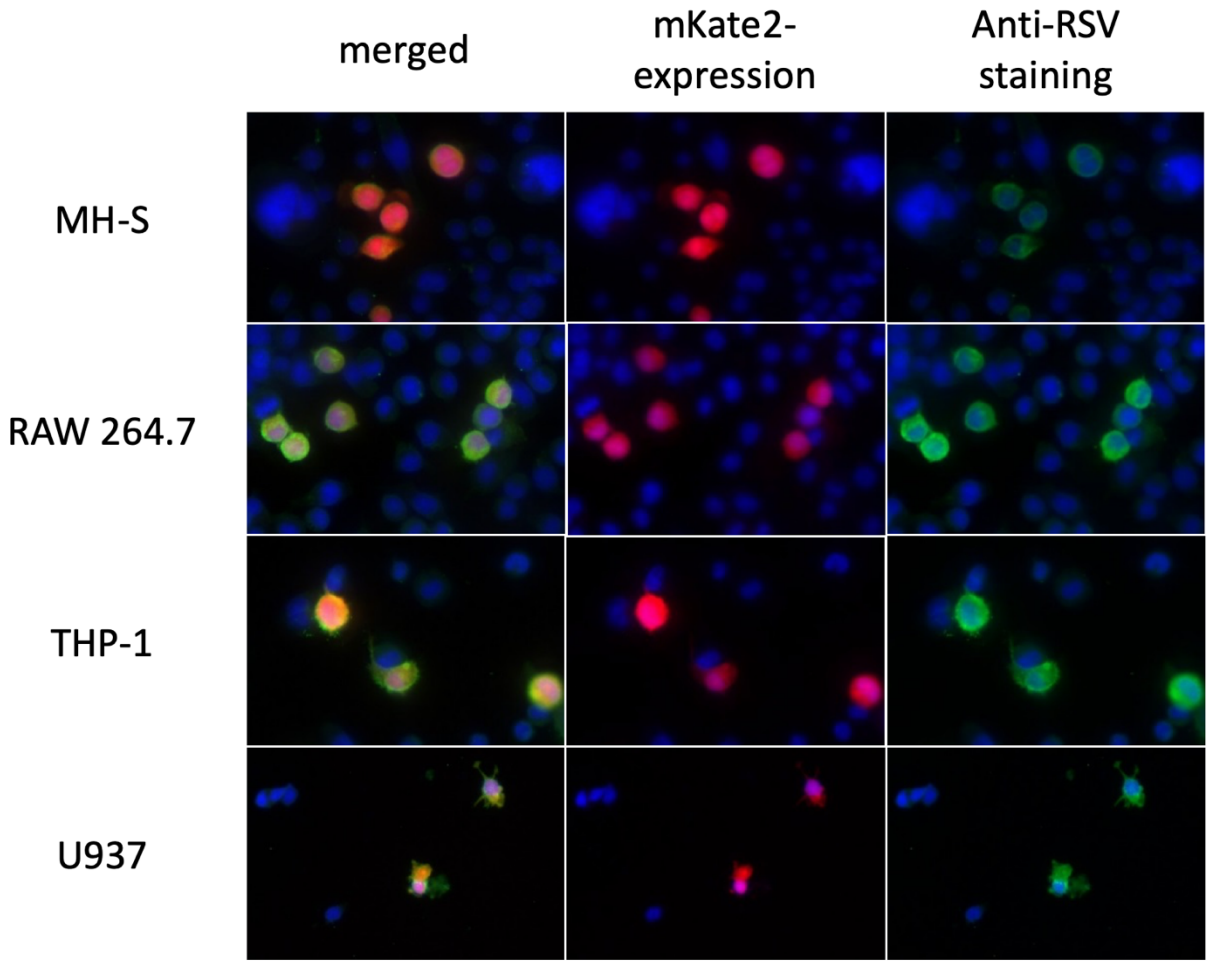
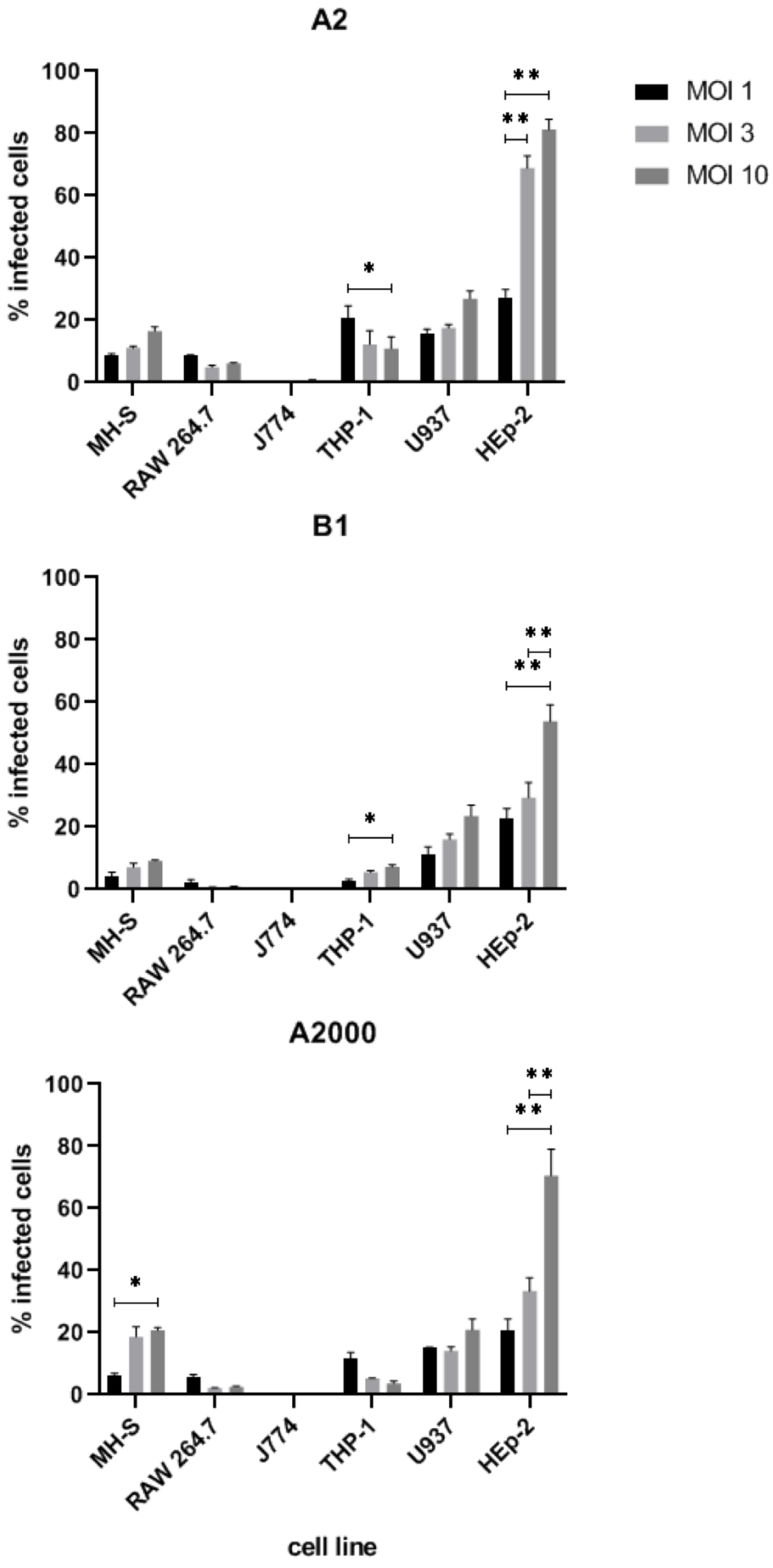
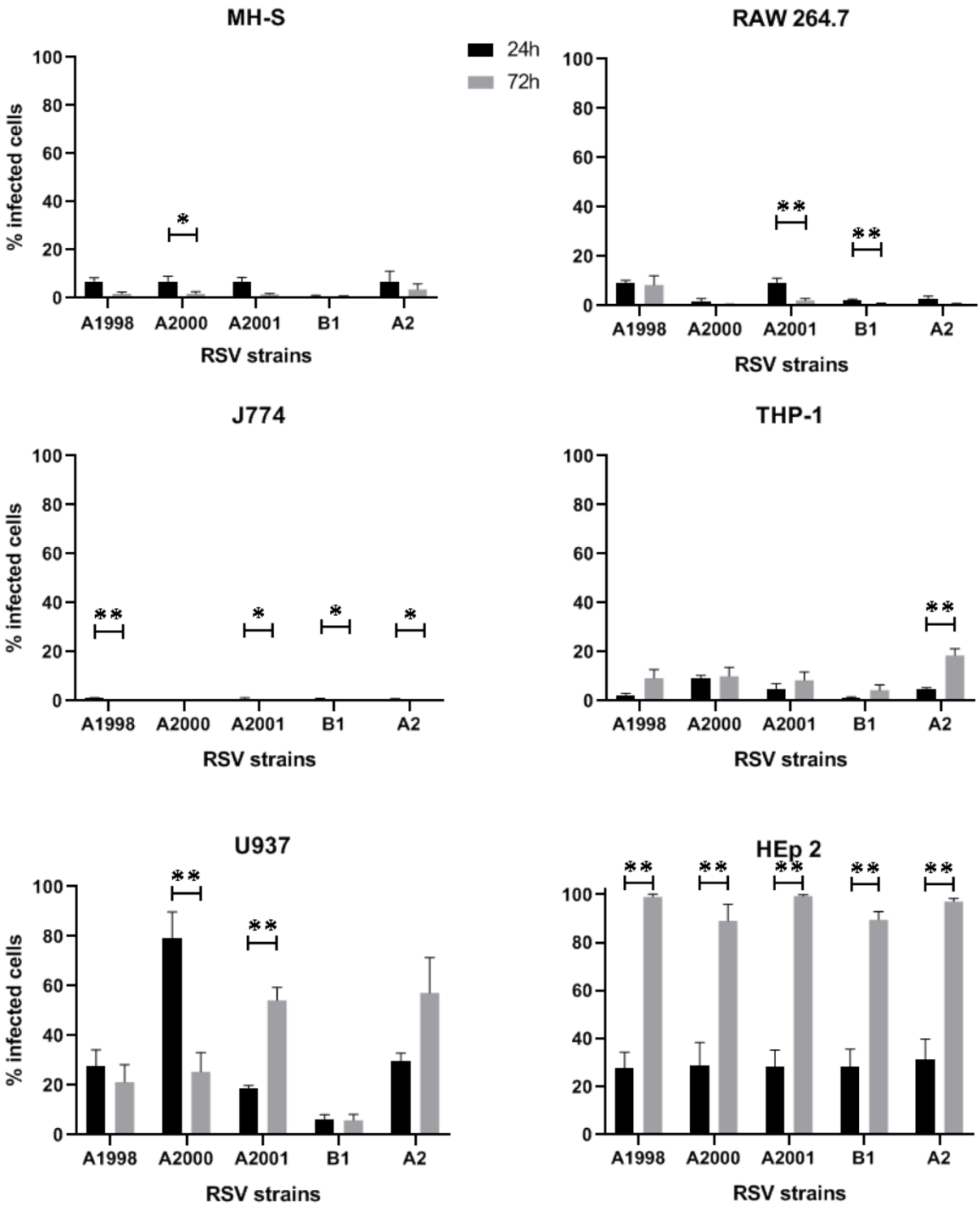
27,4 %
25,5 %
29,2 %
0%

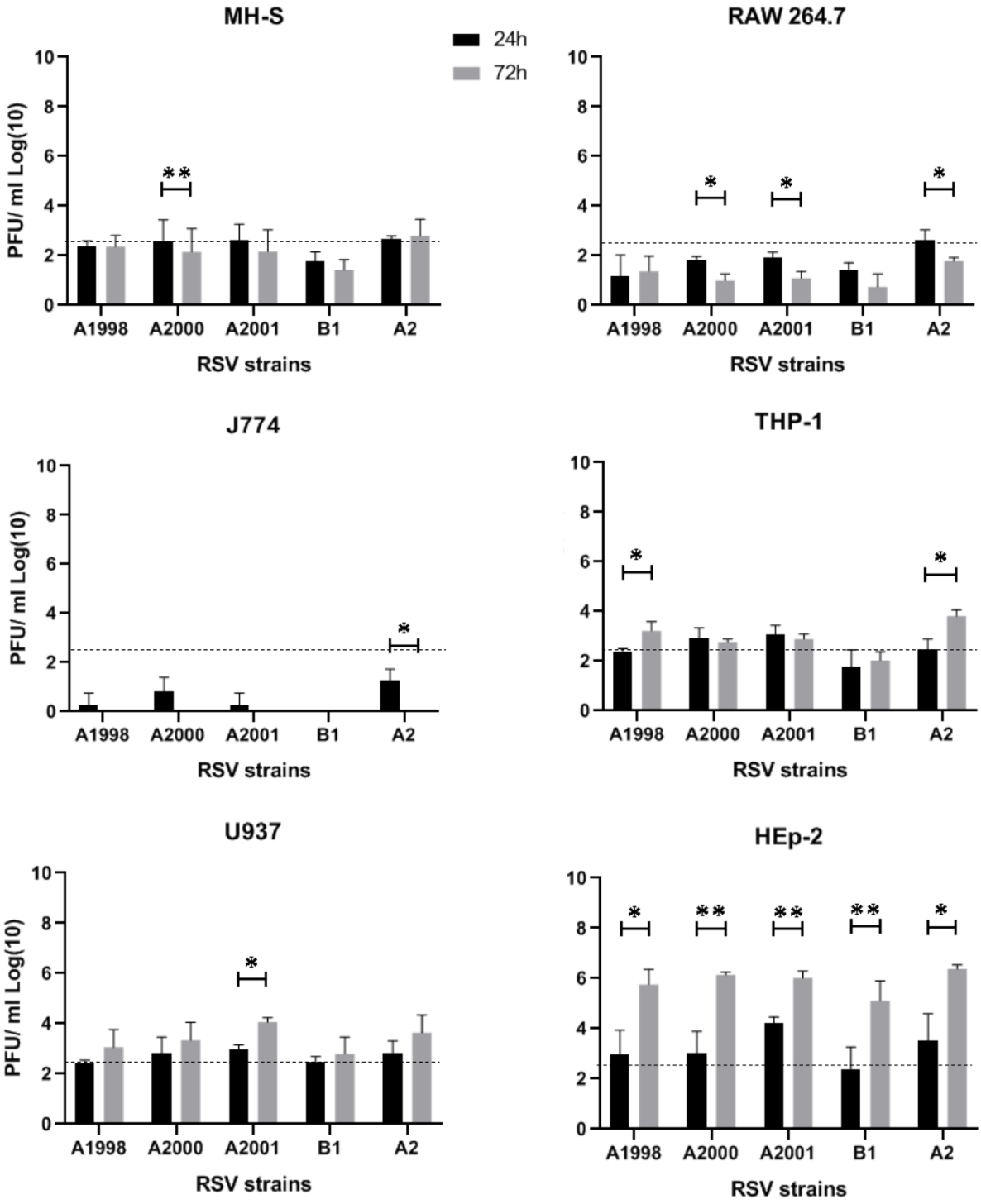

A1998
A2000
A2001
B1
A2
% infected cells
0
10
20
30
40
50
MH-S
RAW 264.7
J774
THP-1
U937
HEp-2
cell line

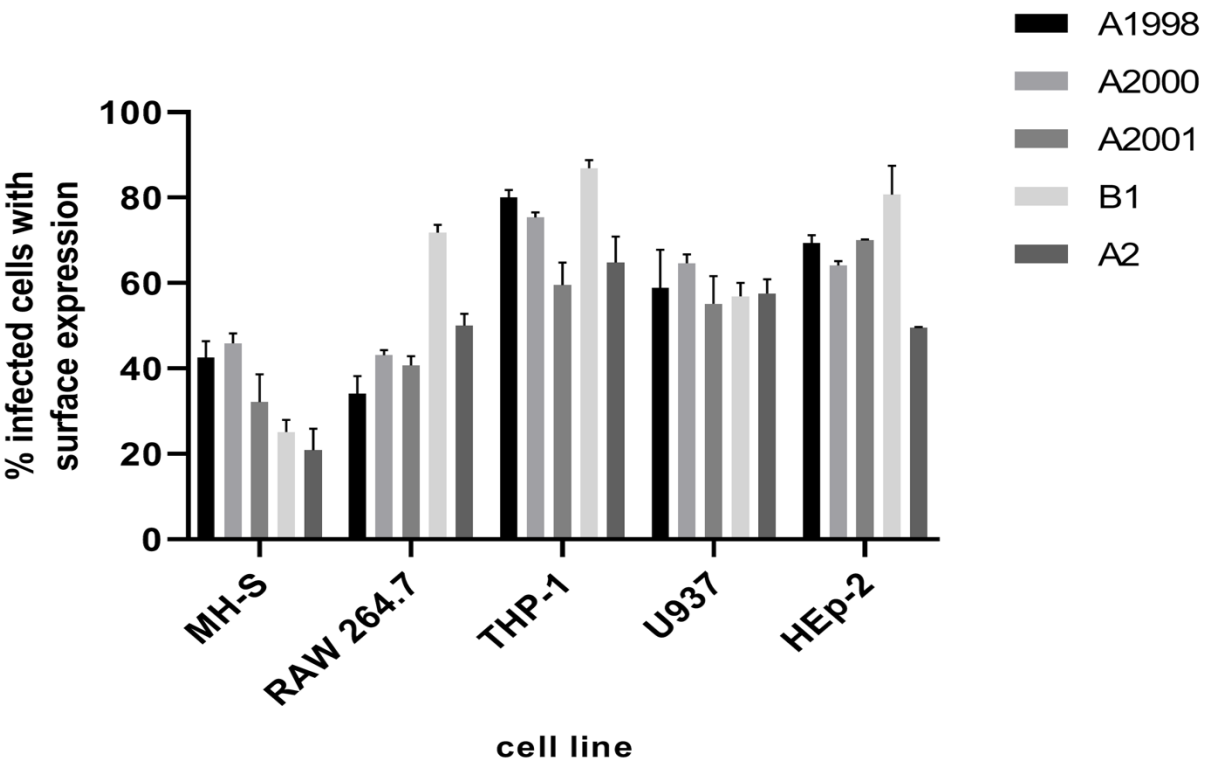

MH-S
RAW 264.7
J774
U937
THP-1
HEp-2
% infected cells
RSV strains
A1998
A2000
A2001
B1
A2


merged
mKate2-expression
Anti-RSV staining
MH-S
RAW 264.7
THP-1
U937


A2
B1
A2000
MOI 1
MOI 3
MOI 10
% infected cells
100
80
60
40
20
0
MH-S
RAW 264.7
J774
THP-1
U937
HEp-2
*
**
cell line


MH-S
RAW 264.7
J774
THP-1
U937
HEp 2
24h
72h
% infected cells
RSV strains
A1998
A2000
A2001
B1
A2
0
20
40
60
80
100
*
**


MH-S
24h
72h
PFU/ ml Log(10)
RSV strains
A1998
A2000
A2001
B1
A2
RAW 264.7
J774
THP-1
U937
HEp-2


A1998
A2000
A2001
B1
A2
% infected cells with surface expression
100
80
60
40
20
0
MH-S
RAW 264.7
THP-1
U937
HEp-2
cell line