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ABSTRACT

Respiratory syncytial virus (RSV) infections remain a major cause of respiratory disease and hospitalizations among infants. Infection recurs frequently and establishes a weak and short-lived immunity. To date, RSV immunoprophylaxis and vaccine research is mainly focused on the RSV fusion (F) protein, but a vaccine remains elusive. The RSV F protein is a highly conserved surface glycoprotein and the main target of neutralizing antibodies induced by natural infection. Here, we analyzed an internalization process of antigen–antibody complexes after binding of RSV-specific antibodies to RSV antigens expressed on the surface of infected cells. The RSV F protein and attachment (G) protein were found to be internalized in both infected and transfected cells after the addition of either RSV-specific polyclonal antibodies (pAbs) or RSV glycoprotein-specific monoclonal antibodies (mAbs), as determined by indirect immunofluorescence staining and flow-cytometric analysis. Internalization experiments with different cell lines, well-differentiated primary bronchial epithelial cells (WD-PBECs) and RSV isolates suggest that antibody-internalization can be considered as a general feature of RSV. More specifically for RSV F, the mechanism of internalization was shown to be clathrin-dependent. All RSV F-targeted mAbs tested, regardless of their epitopes, induced internalization of RSV F. No differences could be observed between the different mAbs, indicating that RSV F internalization was epitope-independent. Since this process can be either antiviral, by affecting virus assembly and production, or beneficial for the virus, by limiting the efficacy of antibodies and effector mechanism, further research is required to determine the extent to which this occurs *in vivo* and how this might impact RSV replication.

IMPORTANCE

Current research into the development of new immunoprophylaxis and vaccines is mainly focused on the RSV F protein since, among others, RSV F-specific antibodies are able to protect infants from severe disease, if administered prophylactically. However, antibody responses established after natural RSV infections are poorly protective against reinfection and high levels of antibodies do not always correlate with protection. Therefore, RSV might be capable of interfering, at least partially, with antibody-induced neutralization. In this study, a process through which surface-expressed RSV F proteins are internalized after interaction with RSV-specific antibodies is described. One the one hand, this antigen–antibody complex internalization could result in an antiviral effect, since it may interfere with virus particle formation and virus production. On the other hand, this mechanism may also reduce the efficacy of antibody-mediated effector mechanisms towards infected cells.

INTRODUCTION

Human respiratory syncytial virus (RSV) is a leading cause of severe lower respiratory tract disease in young children and a major cause in the elderly and immunocompromised patients worldwide (1, 2). Nearly all children are exposed to RSV by two years of age, and prematurity, bronchopulmonary dysplasia and congenital heart disease are risk factors for developing severe RSV disease, including bronchiolitis and pneumonia (1). RSV may also cause significant disease in adults and furthermore re-infection can occur throughout life (2). Despite the discovery of the virus in 1956, no safe and effective vaccine is currently available to control RSV infections (3). Treatment of severe infections is primarily supportive by maintenance of hydration and oxygenation. Palivizumab, a humanized monoclonal antibody, targets a conserved epitope of the RSV fusion (F) protein and is administered prophylactically to high-risk patients (4). Severe RSV disease appears to be linked to an unbalanced and incomplete immune response. Several factors that allow RSV to evade host defense have already been described (2, 5, 6).

RSV belongs to the Pneumoviridae, genus Orthopneumovirus, which is comprised of enveloped viruses with a negative-stranded RNA genome. The 15.2 kb non-segmented genome is comprised of 10 genes that encode 11 proteins. Among these are three surface glycoproteins, the G glycoprotein, the F protein and the small hydrophobic (SH) protein (1). The G protein is responsible for attachment with host cells, which are predominantly ciliated airway epithelial cells (7, 8). Fusion of the viral and cellular membranes is facilitated by the RSV F protein, as is fusion between the membranes of infected cells with adjacent cells, which result in large, multinucleated syncytia. The smaller SH protein is considered to act like a viroporin and increases membrane permeability (5). Of these envelope glycoproteins, only the RSV F protein is indispensable for viral replication *in vitro* (9). It is the most conserved RSV glycoprotein and also

the main target of neutralizing antibodies and vaccine development (10, 11). Initially, the RSV F protein assembles into a homotrimeric, metastable prefusion conformation that rearranges to a highly stable postfusion conformation during fusion of the viral and target cell membrane or spontaneously (12). Six major antigenic sites are currently identified that are located on the prefusion and/or postfusion trimer conformation of the RSV F protein (10, 13-15). Palivizumab, directed to antigenic site II, is the only approved immunoprophylaxis and provided a 55% reduction in RSV-associated hospitalizations in a phase III trial (16). At present, the use of potent neutralizing antibodies directed to other epitopes and/or targets is being extensively studied as alternative approaches for both therapy and prophylaxis. This research is mainly focused on highly potent antibodies that recognize the prefusion RSV F conformation. Three antibodies (5C4, AM22 and D25) were shown to bind the prefusion specific antigenic site Ø, located at the apex of the prefusion trimer (14). Recently, two novel prefusion-specific antibodies, MPE8 and AM14, were characterized and shown to bind antigenic sites III and V, respectively (10, 15, 17). The epitope for MPE8 is located near the binding site of palivizumab in the groove between the helix-turn-helix and the ridge of antigenic site IV on the adjacent protomer. It partially competes with mAbs to sites II, IV and V. This epitope is well-conserved between other pneumoviruses of the *Paramyxoviridae* family (15). Antigenic site V, targeted by AM14, spans from the tip of the β3-β4 hairpin of one protomer to the distal end of antigenic site IV on the adjacent protomer (17).

Internalization of viral envelope proteins expressed on the surface of infected cells is a commonly seen characteristic of viruses, including paramyxoviruses (18-22). For most viruses, the relevance of this process is not yet fully understood. In the case of the Henipavirus fusion proteins, internalization from the surface is essential for proteolytic activation by cathepsin L (19). Also, virus assembly can be affected by the internalization of viral glycoproteins (23). Furthermore, internalization can be important for viral pathogenesis by down-regulation of viral

antigen surface expression and reduced recognition of infected cells by the immune system (20, 24-26). Two different types of internalization have been described previously. Spontaneous endocytosis was observed for many herpesviruses and human immunodeficiency virus (HIV) among others. A second type of internalization is induced by the interaction of specific antibodies with viral proteins expressed on the surface of infected cells, followed by internalization of antibody-antigen complexes in the cell (25, 27, 28). Such viral protein internalization may either result from cross-linking or depend on specific endocytic motifs in the cytoplasmic or transmembrane domains of glycoproteins, such as common tyrosine-based sorting motifs and di-leucine motifs (20, 24, 29, 30).

Previous studies have shown that upon binding of goat anti-RSV polyclonal antibodies (pAbs) to RSV antigens expressed on the surface of infected HEp-2 cells, internalization of these RSV antigen–antibody complexes may occur (31, 32). In this study, we examined this process in the context of a viral infection as well as at the level of individual RSV glycoproteins expressed at the surface of transfected cells. Both strategies demonstrated uptake of RSV antigen–antibody complexes in a time-dependent manner that resulted in a reduction of surface expressed RSV antigens. This process is modulating surface expression of RSV antigens and may affect induction of and recognition by RSV-specific antibodies. Since the RSV F protein elicits the most potent neutralizing antibodies and is currently the most interesting target for therapeutic and prophylactic purposes, this study aimed to characterize antibody-induced internalization of RSV antigens, in particular RSV F, in more detail.

MATERIAL AND METHODS

Cells, virus and antibodies

Human epidermoid carcinoma larynx cell line (HEp-2) and A549 cell line were obtained from ATCC. The cells were grown in Dulbecco's modified Eagle medium (DMEM) and Ham's F-12K (Kaighn's) medium respectively, both supplemented with 10% inactivated fetal bovine serum (iFBS) (Thermo Fisher Scientific). BSR T7/5 cells were a gift of K.K. Conzelmann (Max-von-Pettenhofer-Institut, Munich, Germany) and grown in Glasgow's minimal essential medium (GMEM) supplemented with 10% iFBS and 2% minimal essential amino acids (Thermo Fisher Scientific). Well-differentiated primary paediatric bronchial epithelial cell (WD-PBECs) culture was described previously (33). RSV reference strains A2, B1 and clinical isolate A1998/3-2 were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI resources) and propagated in HEp-2 cells. Recombinant RSV encoding the far-red fluorescent protein monomeric Katushka-2 (mKate2) was recovered as described previously (34). Commercially available goat anti-RSV pAb (Virostat), mouse anti-RSV F IgG (clone 131-2A; Millipore) and mouse anti-RSV G IgG (clone 131-2G; Millipore) were used as reference antibodies. Human reference antiserum was obtained from BEI resources (BEI NR-4020). A panel of RSV F-specific mAbs and their corresponding Fab fragment were provided by J.A. Melero, J.S McLellan, B.S. Graham and C.A.M. de Haan. Secondary antibodies donkey anti-goat IgG, chicken anti-mouse IgG and goat-anti human IgG, conjugated with Alexa Fluor (AF) 488 or 555, obtained from Thermo Fisher Scientific and FITC-conjugated rabbit anti-human IgG (DAKO), were used to visualize the antigens.

Construction and expression of recombinant RSV proteins

Synthesis of the RSV F and RSV G protein was performed by Genscript and delivered in pUC57, a commonly used plasmid for cloning. Restriction enzymes (New England Biolabs) were used to subclone the recombinant sequences in a mammalian expression vector pBudCE4.1 (Thermo

Fisher Scientific). Transfection of the resulting plasmids was performed with Lipofectamine 2000 (Thermo Fisher Scientific) in Opti-MEM (Thermo Fisher Scientific) to obtain surface expression of recombinant RSV proteins. Briefly, BSR T7/5 cells were seeded on coverslips in 24-well plates to be confluent at the time of transfection. Plasmid DNA was mixed with Lipofectamine 2000 and incubated at room temperature during 20 min. Next, the transfection complexes were added to the cells and incubated for 2 h at room temperature. Further incubation at 37°C was performed after adding complete GMEM for 6 h. Finally, the complexes were removed, replaced by complete GMEM and incubated overnight at 37°C.

Antibody-induced internalization assay

Cells were seeded on coverslips in 24-well plates to be subconfluent or confluent at the time of infection or transfection respectively. RSV infection was performed by diluting the virus stock in basal growth medium and subsequently adding the virus suspension to the cells. After 2 h incubation at 37°C, the inoculum was removed and replaced by complete growth medium and further incubated at 37°C. Transfections were performed as described above. After 24 h incubation, RSV-infected or transfected cells were incubated with antibodies against RSV for 1 h at 4°C to allow only attachment of the antibodies. To remove unbound antibody, cells were washed three times with growth medium, followed by incubation at 37°C to start the internalization process. After different time points, cells were fixed with 4% paraformaldehyde (Merck) and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich). To visualize antigen–antibody complexes, cells were stained with appropriate secondary antibodies conjugated with AF 488, AF 555 or FITC. As a control, cells were fixed after the 4°C incubation (T0).

Flow-cytometric analysis of RSV internalization

Surface expression after internalization was quantified by flow cytometry. HEp-2 and A549 cells were seeded in 6-well plates to be subconfluent after 24 h incubation at 37°C or kept in suspension in pre-coated glass vials (Sigmacote®) for 24 h. Infection of the cells was performed as described above. After 24 h, infected cells in 6-well plates were detached by incubation with 1 mM EDTA (Sigma) during 30 min at 4°C and pelleted by centrifugation (210 x g, 10 min, 4°C). The pellet was resuspended in RPMI supplemented with 10% iFBS, then incubated with primary antibodies during 1 h at 4°C and washed to remove unbound antibodies. Cells were kept at 4°C as timepoint 0. The other samples cells were then shifted to 37°C by the addition of warm RPMI medium and further incubated at 37°C during 90 min. To stop internalization, cells were shifted to 4°C for 15 min. Afterwards, the cells were incubated with AF 488-conjugated secondary antibodies for 1 h at 4°C, washed with PBS and analyzed by flow cytometry with a FACSCalibur. Dead cells were excluded by staining the cells with LIVE/DEAD® fixable far-red dead cell stain (Thermo Fisher Scientific). Forward-scattered light (FSC), side-scattered light, the AF 488 (FL-1) and far-red fluorescence signal (FL-4) were stored for further analysis. Mean fluorescence intensity (MFI) was calculated from three independent repeats. The reduction in surface expression was calculated as follows: $100 - [MFI_{90\ min} - MFI_{background}] / [MFI_{0\ min} - MFI_{background}] \times 100$ whereas $MFI_{background}$ is the mean of the fluorescence signal of stained non-infected cells.

Spontaneous endocytosis assay

Infection of subconfluent HEp-2 cell cultures was performed as described above. Surface proteins of the infected cells were biotinylated using EZ-link Sulfo-NHS-SS-biotin (Thermo Fisher Scientific) at 4°C. Then, cells were incubated at 37°C to allow endocytosis to occur. As a control of antibody-induced internalization, one sample was incubated with RSV-specific antibodies.

Biotin was removed from surface proteins by the addition of cleavage buffer (60 mM L-glutathione, 75 mM NaCl, 10 mM EDTA, pH 7.5) for 30 min at 4°C. One sample was neither incubated nor reduced with cleavage buffer to determine the total amount of biotinylated proteins. The efficiency of biotin removal by cleavage buffer was determined by cleavage of a non-incubated sample. After lysis of the cells with RIPA lysis buffer (Millipore), biotinylated proteins were immunoprecipitated using Streptavidin Mag Sepharose (Sigma) and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing conditions. The presence of RSV F proteins was detected by transferring the gel to a PVDF membrane and subsequent incubation with RSV F specific mAb.

Internalization inhibition assays

To inhibit antibody-induced internalization, two strategies were performed as previously reported (35). First, inhibitors were used in different concentrations before and during the internalization assay. Amantadine and nystatin, purchased from Sigma, and myristoylated dynamin inhibitory peptide (DIP), purchased from Tocris Bioscience, were diluted in their solvent prior to dilution in cell-culture medium. After 1 h pretreatment of the cells with the inhibitors at 37°C, fresh inhibitor was added to the cells together with antibodies to induce internalization. As a control for each drug, cells were incubated with the corresponding solvent of the inhibitor. Controls were applied to confirm the effectiveness of the drugs, including biotinylated transferrin for clathrin-mediated endocytosis and FITC-labelled BSA for caveolae-mediated endocytosis. The biotinylated transferrin was visualized with streptavidin-FITC. Second, inhibition of internalization was performed using plasmids encoding dominant negative (DN) proteins that inhibit clathrin-, caveolae- or dynamin-mediated endocytosis. These constructs and their wild-type (WT) counterparts were originally provided by A. Benmerah (36, 37), A. Helenius (38, 39) and M.

McNiven (40, 41) respectively and further modified as described before (42). Co-transfection of BSR T7/5 cells was performed by diluting equal amounts of DNA of the RSV F protein and the DN or WT protein. WT and DN dynamin 2(aa) with a C-terminal enhanced GFP (eGFP) tag, were used to inhibit dynamin-dependent endocytosis. To inhibit clathrin-mediated endocytosis, the DN mutant of the protein Eps15 (DIII), essential for the docking of adaptor protein-2 during assembly of clathrin-coated pits, was used (42). This EGFP-tagged mutant has a deletion at the Eps15 homology and coiled-coil domains. A construct with a supplementary deletion of the AP-2-binding site served as a negative control (D3Δ2). WT and DN caveolin-1 constructs were used for caveolae-mediated endocytosis.

Microscopic analysis

All high-resolution images of monolayer cultures were obtained using Apotome 2 with a Axio Observer inverted microscope with a compact light source HXP 120C (Zeiss) and using a Nikon Eclipse Ti-E inverted microscope attached to a microlens-enhanced dual spinning disk confocal system (UltraVIEW VoX; PerkinElmer, Zaventem,Belgium) equipped with 405 and 488 nm diode lasers for excitation of blue and green fluorophores, respectively. The images were obtained with ZEN 2012 and Volocity 3D Image Analysis Software.WD-PBEC imagese were obtained with a Nikon eclipse 90i inverted microscope.

Statistical analysis

Data are presented as means (±SD) of three independent repeats and were analyzed by a student's t-test using GraphPad Prism 6. P values <0.05 were considered statistically significant.

RESULTS

Antibody-induced internalization of cell surface-expressed RSV antigens

To evaluate antibody-induced internalization of RSV antigens, RSV-infected HEp-2 and A549 cells were incubated with polyclonal goat anti-RSV IgG for 1 h and subsequently fixed and stained with AF488 donkey anti-goat IgG. At time point 0, membrane staining was observed for both cell types and none of the infected cells showed vesicles in their cytoplasm (Fig.1A). After 1 h incubation at 37°C, multiple intracellular vesicles were present in the cytoplasm of the cells and a reduction in surface expression was observed. No intracellular vesicles were observed in non-infected cells. Isotype control antibodies and secondary antibodies only were used to confirm that the internalization process is specifically induced by the interaction of RSV-specific antibodies with RSV antigens (data not shown). In addition, the kinetics of antibody-induced internalization was analyzed. Fig.1B shows the kinetics of the percentage of cells positive for intracellular RSV antigen–antibody complexes, which reached a maximum at 60 min and 90 min for HEp-2 cells and A549 cells, respectively. Similar observations were made based on the number of internalized vesicles (Fig.1C). By flow-cytometric analysis, the surface bound antibodies were measured before and after induction of internalization to allow a more quantitative measurement of internalization (43, 44). For HEp-2 cells and A549 cells, the mean fluorescence intensity (MFI) of surface RSV proteins after the addition of antibodies was reduced to 44% and 40%, respectively, showing that internalization occurs, but that not all RSV proteins are internalized (Fig.1D). Taken together, antibody-induced internalization of RSV antigens is time-dependent, but not all molecules are internalized upon binding of pAbs. Addition of increasing concentrations of pAbs resulted in rising levels of internalized vesicles between 0.001 and 0.01

mg/ml, followed by a slower increase between 0.01 and 0.3 mg/ml (Fig. 1E). At a concentration of 1 mg/ml, the internalization efficiency decreased.

Two major antigenic subgroups (A and B) have been described for RSV. With regards to the RSV surface proteins F and G, an amino acid identity of 91% and 51%, respectively, exists between the subgroups (45). After infection with the RSV B1 reference strain, internalization of RSV-specific pAbs in complex with RSV surface antigens was also observed (Fig.2A). Additionally, a clinical strain (A1998/3-2) was evaluated and numerous intracellular vesicles were also observed, and surface expression of RSV proteins was reduced compared to time point 0 (Fig.2A). These findings suggest that internalization of RSV surface antigens is a general feature of RSV. In addition to polyclonal goat anti-RSV antibodies obtained after immunization, a human anti-RSV reference serum (obtained after natural RSV infection) was also evaluated. After a 60 min incubation at 37°C, internalization and antigen–antibody complexes were detected in the cell cytoplasm, and some complexes remained at the surface of infected HEp-2 cells (Fig.2B). Finally, the internalization process was also analyzed in RSV-infected well-differentiated primary bronchial epithelial cells (WD-PBECs). After 120 min incubation with human RSV-specific antiserum, internalized RSV antigen–antibody complexes were observed in more than 80% of the RSV-infected cells (Fig.3A,B).

Determination of the cell-surface expressed RSV proteins involved in the internalization process

Since RSV F and G proteins are the two major surface antigens and the only RSV proteins that induce neutralizing antibodies, the involvement of the respective RSV surface glycoproteins was identified. For this purpose, RSV glycoprotein-specific mAbs were used. For both surface proteins and in both cell types, internalization and intracellular vesicles were observed (Fig.4A).

303 Analysis of internalization in BSR T7/5 cells transfected with the individual RSV F or G proteins
304 was performed to investigate their respective roles in this process. Internalization of RSV
305 antigens was also observed after RSV infection of BSR T7/5 cells and incubation with RSV-
306 specific antibodies, showing that this process also occurs in these cells (data not shown). For both
307 proteins, multiple internalized antigen–antibody complexes were present in vesicles in the
308 cytoplasm of cells transfected with the RSV F or G protein and after incubation with either RSV-
309 specific pAbs or RSV glycoprotein-specific mAbs (Fig.4B). These observations show that
310 internalization of both RSV surface proteins occurs independent of other viral proteins.
311
312 **Internalization of surface-expressed RSV F proteins is clathrin-mediated and mainly**
313 **triggered after antibody-induced cross-linking of the RSV F protein**
314 As mentioned earlier, RSV F is the major target of neutralizing antibodies and plays a central role
315 in the development of new immunoprophylaxis and vaccine strategies. Therefore, the
316 characteristics of RSV F internalization were studied in more detail. The endocytic route through
317 which RSV F-antibody complexes are internalized, was investigated by using inhibitors that
318 block different mechanisms of endocytosis. DIP is an inhibitor of the GTPase dynamin that
319 blocks the binding of dynamin to amphiphysin (46). Addition of this peptide during
320 internalization resulted in a reduction of approximately 50% of internalized vesicles (Fig.5A).
321 Inhibition by dynasore, an inhibitor that blocks dynamin by disturbing the plasma membrane
322 cholesterol homeostasis (47), resulted in similar reductions (data not shown). In addition, BSR
323 T7/5 cells expressing a recombinant RSV F protein were co-transfected with dominant negative
324 (DN) proteins to inhibit a specific internalization process. By using an eGFP-tagged DN mutant
325 of dynamin 2(aa) in RSV F transfected BSR T7/5 cells, a significant reduction of internalized
326 vesicles (62.01%) was observed in cells transfected with DN dynamin compared to WT dynamin

transfected cells (Fig.5B,G). Both results show the involvement of a dynamin-dependent mechanism. Since GTPase dynamin is recruited to both clathrin-coated pits and caveolae, further distinction was made between these endocytic routes (48). Amantadine was used to test the dependence of clathrin and resulted in a dose-dependent reduction of intracellular vesicles with a maximum of 74.09% (Fig.5C). By using a DN mutant of Eps 15 (DIII) and the control plasmid DIIIΔ2 the role of clathrin was further analyzed. DIII transfected cells showed a significant reduction in internalization compared to DIIIΔ2 transfected cells (Fig.5D,G). No difference in the amount of internalized vesicles was observed after treatment of the cells with nystatin, a sterol-binding agent which disrupts caveolae (Fig.5E). These results were confirmed in RSV F transfected cells co-transfected with a GFP-tagged DN mutant of caveolin-1, which showed no significant differences in internalization of RSV F compared to cells transfected with the control plasmid, an eGFP-tagged WT caveolin-1 (Fig.5F,G).

In addition, we investigated the extent to which spontaneous endocytosis occurs for RSV proteins by labelling the surface proteins of infected cells with a membrane impermeable biotinylation reagent, followed by an internalization assay. Cell surface biotinylation was efficient (Fig.6E) and the addition of cell-impermeable glutathione removed almost all biotin from the cell surface (Fig.6D). Antibody-induced internalization of biotinylated surface proteins resulted in protection from glutathione-mediated biotin cleavage. A difference in the amount of intracellular (biotinylated) RSV F proteins could be observed between internalization induced by antibodies (Fig.6C) and spontaneous endocytosis in the absence of antibodies (Fig.6B), confirming that the majority of RSV F internalization is triggered by antibodies.

Intact monoclonal IgG antibodies can cross-link surface-expressed antigens and stimulate their internalization (49, 50). To determine whether cross-linking of RSV F proteins is required for internalization, monovalent Fab fragments of RSV F-specific mAbs were compared with the

intact mAbs (Fig.7A). Internalization of RSV F proteins was significantly reduced when induced by the monovalent Fab fragments (Fig.7B), indicating that cross-linking plays a role in efficient RSV F internalization.

Internalization of surface RSV F proteins is triggered by binding of different neutralizing RSV F epitope-specific mAbs

Different neutralizing epitopes on the RSV F protein have already been identified and mAbs directed against these epitopes are available (10, 13-15). Since mAbs recognizing different epitopes of the same protein could have different effects on internalization, the epitope-dependence of RSV F internalization was analyzed (51). The amount of internalized RSV F-mAb complexes was determined by flow-cytometric analysis. After shifting RSV-infected HEp-2 cells in suspension to 37°C, anti-RSV F mAbs attached to surface RSV F proteins were internalized and the amount of internalization was quantified by a reduction of surface expression. A reduction in MFI was seen for all mAbs, compared to the MFI of cells that were kept at 4°C and did not undergo internalization. The amount of internalization, as calculated by the reduction of surface fluorescence, ranged from 31 to 57% for mAb 131-2A and AM22, respectively. Overall, no major differences could be observed between prefusion RSV F-specific mAbs or mAbs that bound both pre- and postfusion RSV F (Table 1). The results also showed that not all RSV F molecules are internalized upon stimulation with the mAbs. These findings were confirmed by analysis with fluorescence microscopy where surface expression of RSV F was still observed after induction of internalization (data not shown).

DISCUSSION

375 Our results show the potential of RSV-specific antibodies and human sera from RSV-infected
376 patients to trigger internalization of RSV antigens expressed on the surface of infected cells.
377 These findings were clearly documented by confocal microscopic analysis and flow cytometry,
378 and observed in different epithelial cell lines (HEp-2, A549 and BEAS-2B cells (52, 53). In
379 addition, WD-PBEC cultures were used to confirm that this process also occurs in a more
380 representative model of primary airway epithelial cells. The amount of intracellular vesicles
381 increased with increasing time of incubation and reached a plateau after 60 to 90 min in
382 monolayer cells. Furthermore, surface-expressed glycoproteins of different RSV strains,
383 including both reference strains (A2 and B1) and a clinical isolate (A1998/3-2), were shown to be
384 susceptible to antibody-induced internalization (54). Taken together, our findings suggest that the
385 internalization process is a general feature of RSV-infected cells. Using inhibitors and plasmids
386 encoding dominant-negative proteins of endocytic pathways, the internalization was shown to be
387 clathrin-dependent. However, since complete inhibition of RSV F internalization was not
388 achieved, other mechanisms might be involved or could be induced upon blocking a specific
389 pathway (55). To exclude the latter possibility, combinations of inhibitors were tested but did not
390 show any difference compared to treatment with a single inhibitor (data not shown) (56).
391 Additionally, it was shown that RSV F internalization is mainly triggered upon binding of
392 antibodies and is not merely spontaneous internalization, a well-described feature of several
393 paramyxoviruses (18, 19, 21, 23). The results of a biotin internalization assay showed
394 internalization of RSV F proteins after Ab-triggering. Only a weak signal was observed in the
395 absence of antibodies to induce internalization, which might be a consequence of membrane
396 turnover or residual non-cleaved proteins. Interestingly, a reduction in the molecular weight of
397 the RSV F protein was observed upon internalization. A possible explanation is that upon
398 internalization, an event occurs which cleaves the disulphide bridge between F1 and F2 subunits.

399 This can be mediated by the presence of enzymes in the endo-lysosomal system that reduce
400 disulphide bonds and would thus also cleave the F1-F2 bonds (57, 58). Since the mAb used in
401 this experiment is specific for F1, this cleavage will result in detection of a lower molecular
402 weight band on WB, corresponding to the F1 subunit. In addition to binding, antibody-induced
403 cross-linking is most likely needed since we observed that RSV F protein-specific, monovalent
404 Fab fragments were not efficient in inducing internalization. Furthermore, at the highest Ab
405 concentration tested, internalization was induced less efficiently. This could indicate that with
406 high antibody concentrations, cross-linking of RSV F proteins does not efficiently occur because
407 every F protein is bound by a different antibody and thereby cross-linking induced internalization
408 signals would be lost.
409 Previous work showed that internalization of surface-expressed viral glycoproteins can be
410 influenced by interactions with other viral proteins expressed in infected cells. For the Suid
411 herpesvirus I glycoproteins gB and gD, antibody-induced cross-linking was shown to be required
412 for efficient internalization in infected monocytes (22). In contrast, viral core proteins of measles
413 virus are known to regulate the expression of viral glycoproteins and may inhibit internalization
414 during infection to promote virus assembly (18). In our work, internalization was observed in
415 cells transfected with a single RSV protein as well as in RSV-infected cells, indicating that RSV
416 F internalization occurs independently and is not affected by the expression of other viral
417 proteins. How exactly RSV F activates internalization upon antibody binding is not clear. For
418 some viruses, it was shown that internalization can depend on specific endocytic motifs in
419 cytoplasmic and/or transmembrane domain of the viral protein, such as common tyr-based motifs
420 and di-leu motifs (18, 29, 30, 59). For other viruses, it is less clear that internalization depends on
421 specific motifs and internalization may result from cross-linking. Analysis of the cytoplasmic tail
422 of the RSV F protein did not reveal any known amino acid motifs involved in internalization.

Furthermore, analysis of antibody-induced internalization using a mutant RSV F protein lacking the cytoplasmic tail, only resulted in approximately 30% reduction of internalization (data not shown). This suggests that for RSV, specific amino acid motifs are not involved, and that cross-linking is a major driver for internalization, consistent with our finding that monovalent Fab fragments cannot efficiently induce internalization.

Several neutralizing RSV F-specific mAbs are described (Table 1). For some mAbs, it is shown that neutralization results from blocking virus fusion with host cell membranes by fixing RSV F proteins in their prefusion conformation (14, 60). Interestingly, their neutralizing activity may not only be directed against virions but also against virus-infected cells since cell-to-cell fusion can be inhibited by mAbs like palivizumab and motavizumab (60). In this study, flow-cytometric analysis showed that all RSV F-specific mAbs tested had the ability to decrease surface expression of RSV F proteins, which may impact cell-to-cell fusion. While this internalization may not affect cell-to-cell fusion for mAbs that directly block the RSV F fusion activity, it can still affect cell-to-cell fusion indirectly for mAbs that do not interfere with the RSV F fusion activity. As previously shown, different epitope recognition by mAbs could change the internalization pattern of the target antigen (51). However, we observed no differences between the different mAbs, all specific for one of the 6 known RSV F epitopes. These findings suggest that the antibody-induced internalization process of RSV F is not epitope-dependent.

Reinfection with RSV can occur throughout life, indicating that RSV antibody responses only partially provide protection and only for a limited period of time (2, 61). Prophylactic palivizumab is able to prevent severe RSV-induced respiratory tract disease in most, but not in all patients. The cause of the partial failure of this immunoprophylaxis remains unknown, but could be attributed to variations in the dose of inoculum, the efficiency of Ab transfer to the airways and the size of the airways. The process we observed may also affect the activity of anti-F

antibodies if the internalization strongly decreases the amount of RSV-specific antibodies. Presumably, this decrease will not be sufficient to impair the neutralization of free virus particles. Furthermore, the process we observed may also result in an antiviral effect, since internalization of the F protein could interfere with the formation of virus particles and spreading of the virus would thus be restricted already early in infection. On the contrary, interference in effector-mediated destruction of virus-infected cells by this process is more likely. In HIV-1 and SIV-infected cells, internalization of Env proteins provided protection from elimination by antibody-dependent cell-mediated cytotoxicity. Based on these findings, it was suggested that the efficacy of antibody-based therapeutics and HIV-1 vaccines could be improved by disturbing Env internalization (63). For herpesviruses, a similar observation was made (25). Surface expressed virus proteins were internalized by addition of specific antibodies, and antibody-dependent complement-mediated cell lysis was reduced approximately by 50% in infected cells upon antibody-induced internalization (25). Our results showed a remarkable decrease of surface-expressed F proteins after internalization, yet a portion remained on the cell surface. In this regard, further research is needed to elucidate whether there is sufficient internalization to protect RSV-infected cells from antibody-mediated effector responses.

In conclusion, this study describes a mechanism by which the RSV proteins expressed on the surface of infected cells are removed from the cell surface, together with RSV-specific antibodies, by internalization. Whether this process affects the activity of RSV F-specific antibodies and also has consequences for the *in vivo* replication and immune response remains to be elucidated.

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FIGURE LEGENDS

FIG 1 Antibody-induced internalization of RSV antigens. (A) HEp-2 cells and A549 cells were infected with RSV A2 for 24 h and then incubated with a pAb goat anti-RSV antibody bound at 4°C and then shifted to 37°C for 1 h to induce internalization. Afterwards the cells were fixed, permeabilized and stained with AF 488 donkey anti-goat IgG (green). Nuclei were visualized with DAPI (blue). Images represent a single confocal z-section through the middle of the cell. (B) Kinetics of the percentage of cells with internalized RSV antigen–antibody complexes for the indicated time points. (C) Kinetics of the amount of internalized vesicles in positive cells for the indicated time points. (D) Flow-cytometric analysis of RSV internalization. RSV-infected HEp-2 and A549 cells were kept in suspension during the induction of internalization and staining of the cells. The level of the remaining surface expressed RSV antigens after internalization is expressed as MFI relative to T0. (E) Effect of increasing concentrations of pAb goat anti-RSV antibodies on internalization. For each antibody concentration, the amount of internalized vesicles was quantified in 50 positive cells. Data represents the means (± SD) of 3 independent repeats.

FIG 2 Antibody-induced internalization of RSV antigens with different RSV strains and different serum. (A) HEp-2 cells were infected with RSV reference strain B1 or the clinical strain A1998/3-2 during 24 h. Goat anti-RSV pAbs were bound at 4°C (0 min) and internalization was induced at 37°C (60 min). (B) Human reference antiserum was used to induce internalization in RSV A2 infected HEp-2 cells. The cells of A and B were fixed, permeabilized, stained with AF 488 donkey anti-goat IgG and donkey anti-human IgG (green) respectively, and stained with DAPI (blue).

FIG 3 Antibody-induced internalization of RSV antigens in WD-PBECs. WD-PBEC cultures were infected with RSV/A2 mKate (MOI=3) in duplicate. At 72 h post infection, cultures were incubated with human sera for 1 h at 4°C, washed and then transferred to 37°C. Cultures were fixed at 0, 90 and 120 min with 4% paraformaldehyde, permeabilized and incubated with anti-human IgG FITC secondary for 1 h at 37°C. Images for data presented in A-C were taken using a Nikon Eclipse 90i microscope. Images for D were taken with a Leica SP5 confocal microscope. (A) 100 mKate positive RSV-infected cells at each time point in duplicate examined for evidence of internalized antibody using. (B) Representative images at each time point blue (DAPI), red (mKate), green (anti-human IgG 488 antibody). (C) Mock-infected cells incubated with sera for 90 or 120 min and stained with the anti-human IgG 488 antibody. (D) En face and corresponding orthogonal sections with blue (DAPI), green (anti-human IgG 488 antibody). The confocal settings and gain were kept constant for all conditions.

FIG 4 Antibody-induced internalization of RSV surface glycoproteins F and G. (A) RSV infection of HEp-2 and A549 cells during 24 h was followed by incubation of the cells with anti-RSV F (clone 131-2A) or anti-RSV G (clone 131-2G) specific mouse mAbs for 1 h at 37°C. (B) RSV F and RSV G transfected BSR T7/5 cells were incubated with pAb goat anti-RSV or the corresponding mAbs during 1 h. At the indicated time points, the cells were fixed, permeabilized and stained with appropriate AF 488-conjugated secondary antibodies (green) and DAPI (blue). Images were acquired by fluorescence microscopy**.**

FIG 5 Effect of inhibitors and DN proteins on antibody-induced RSV F internalization. RSV-infected HEp-2 cells were incubated with mAb 131-2A in the presence of different concentrations of endocytic inhibitors, DIP (A), amantadine (C) and nystatin (E). After 90 min incubation, cells were fixed, permeabilized and stained. The amount of intracellular vesicles was

quantified and expressed as percentage relative to the number of vesicles in the absence of the inhibitor. BSR T7/5 cells were co-transfected with both RSV F (red) and DN proteins (dynamin 2 (B), Eps15 (DIII) (D) and caveolin-1 (F)) (green). For each DN protein, also a control construct was used (WT dynamin 2 (B), inactive Eps15 (D3Δ2) (D), WT caveolin-1 (F)). After induction of internalization and staining of the cells, the amount of internalized vesicles was determined by fluorescence microscopy. Data represents the mean (±SD) of three replicates. (*, $P < 0.05$).

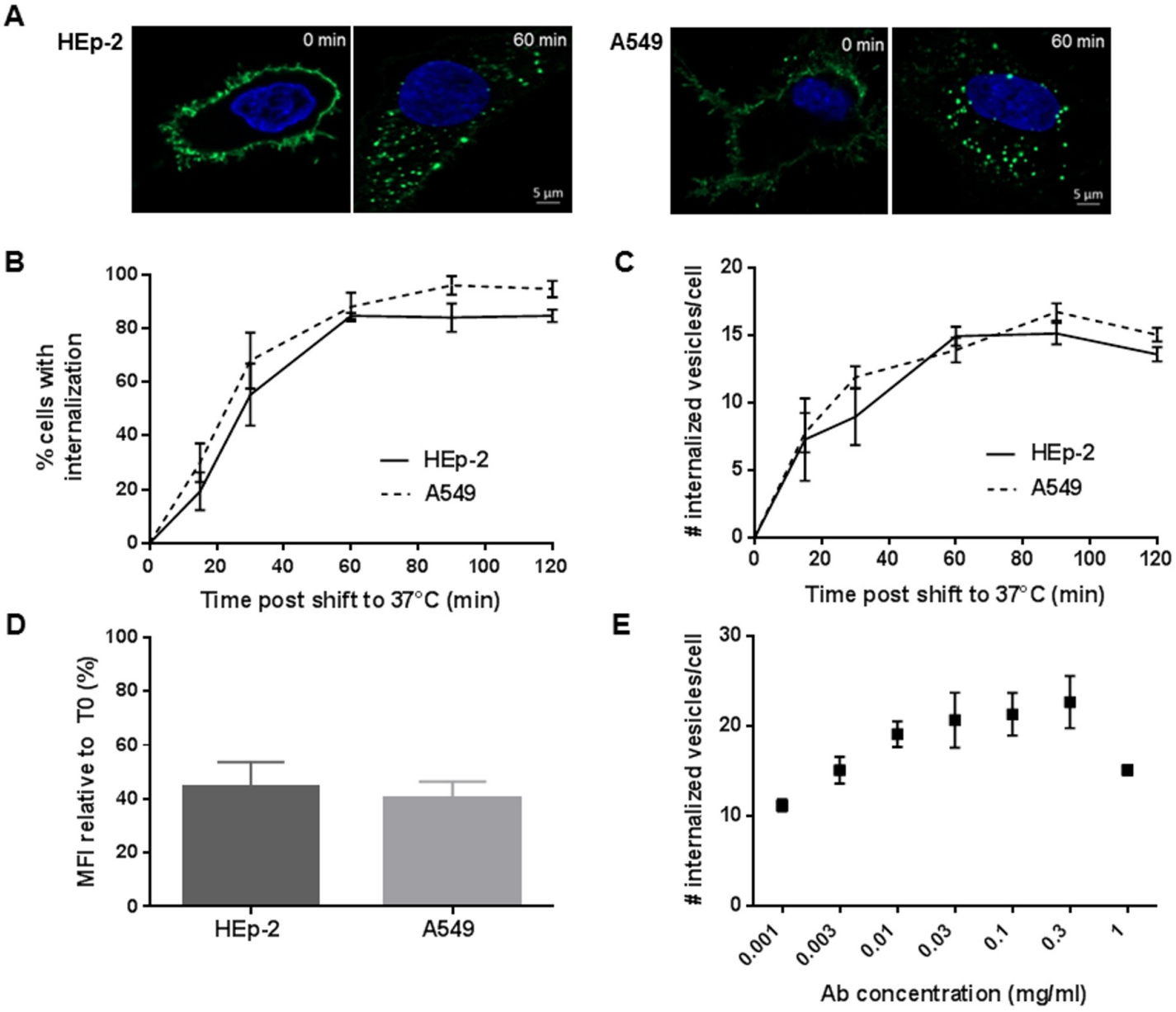
FIG 6 Western blot analysis of a biotin internalization assay of RSV F proteins. RSV-infected and non-infected HEp-2 cells were biotin labeled using a membrane impermeable biotinylation reagent. The cells were shifted with or without antibodies to 37°C during 90 min to allow endocytosis. Non-internalized biotinylated surface proteins were removed by cleavage with glutathione, while internalized proteins were protected from biotin removal. After cell lysis, biotinylated proteins were immunoprecipitated using Streptavidin Mag Sepharose, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing conditions and detected with RSV F specific antibodies. (A) Sample of non-infected cells. (B) Internalized RSV F proteins after incubation at 37°C. (C) Internalized RSV F proteins after incubation with RSV F specific antibodies. (D) Amount of biotinylated surface RSV F proteins after cleavage with glutathione. (E) Total amount of biotinylated surface RSV F proteins before 37°C incubation step. A representative blot of a duplicate experiment is shown.

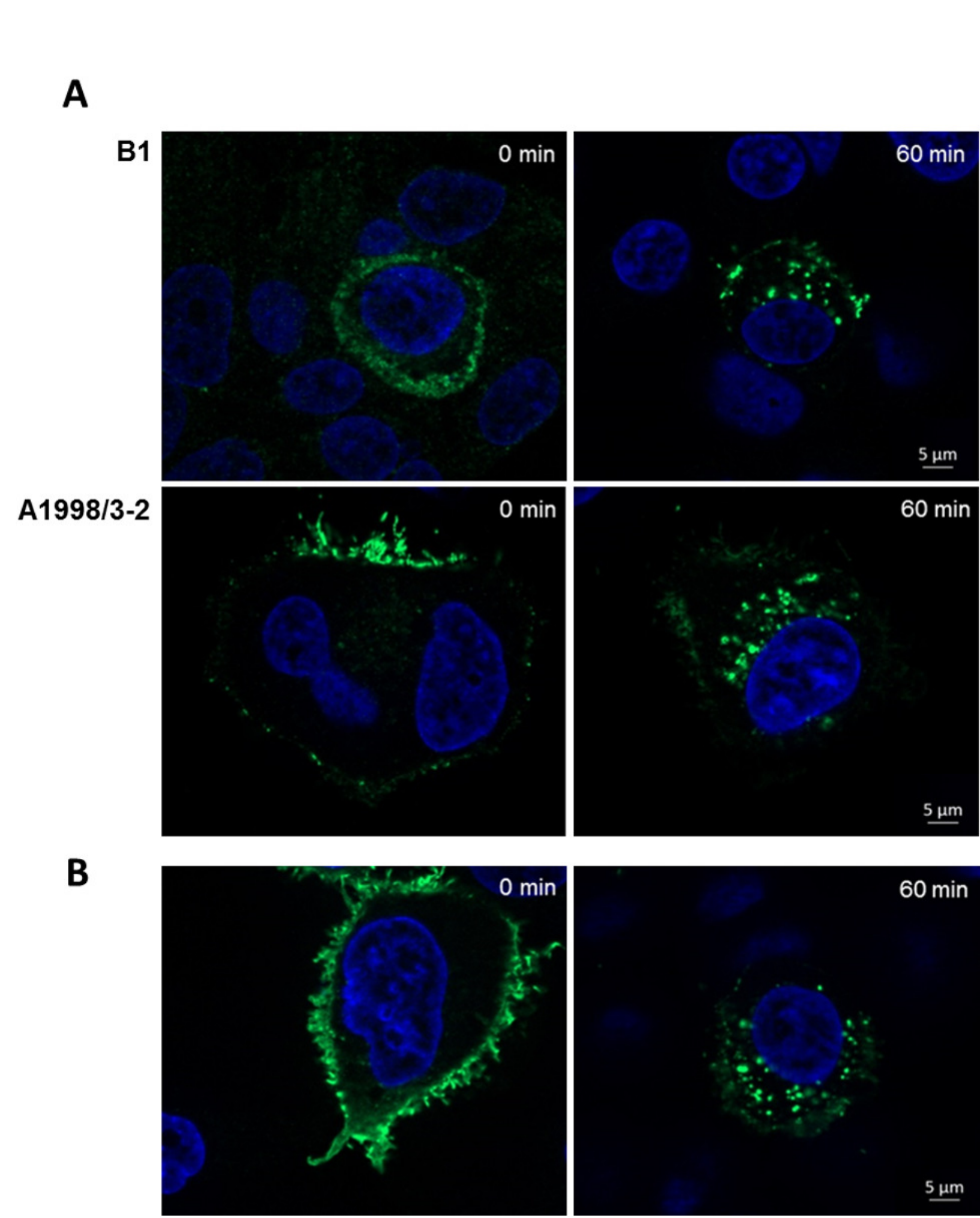
FIG 7 Internalization of Fab fragments. RSV-infected HEp-2 cells were incubated with RSV F specific mAbs D25, AM14, 5C4, MPE8 or corresponding monomeric Fab fragments at the same concentration during 90 minutes to induce internalization. Afterwards the cells were fixed, permeabilized and stained with AF488 human anti-goat IgG or AF488 chicken anti-mouse IgG (green). Nuclei were visualized with DAPI (blue). The amount of internalized vesicles was

quantified in 50 positive cells. Results are shown as means (±SD) of three independent replicates. (*, $P < 0.05$).

TABLE 1 The capacity of different RSV F-specific mAbs to induce internalization. RSV-infected HEp-2 cells in suspension were incubated with the indicated anti-RSV F mAbs during 1 h at 4°C followed by a shift to 37°C to induce internalization of the attached antibodies. After staining the cells with secondary antibodies, the mean fluorescence intensity (MFI) was measured by flow cytometry. The reduction is expressed as percentage relative to T0 (100%). Data represents the mean of 3 independent repeats.

RSV F-specific mAb	% reduction ±SD	RSV F antigenic site	RSV F conformation	Reference
131-2A (murine)	31 ± 9	I	Post (±pre)	(64)
101F (murine)	47 ± 15	IV	Pre and post	(13)
palivizumab	40 ± 18	II	Pre and post	(4)
motavizumab	34 ± 17	II	Pre and post	(65)
AM22	57 ± 2	Ø	Pre	(14)
D25	50 ± 16	Ø	Pre	(14)
5C4 (murine)	39 ± 14	Ø	Pre	(14)
MPE8	44 ± 9	III	Pre	(15)
AM14	42 ± 17	V	Pre	(17)


A
HEp-2
0 min
60 min
5 µm
A549
0 min
60 min
5 µm
B
% cells with internalization
HEp-2
A549
Time post shift to 37°C (min)
C
internalized vesicles/cell
HEp-2
A549
Time post shift to 37°C (min)
D
MFI relative to T0 (%)
HEp-2
A549
E
internalized vesicles/cell
0.001
0.003
0.01
0.03
0.1
0.3
1
Ab concentration (mg/ml)


A
B1
0 min
60 min
5 µm
A1998/3-2
0 min
60 min
5 µm
B
0 min
60 min
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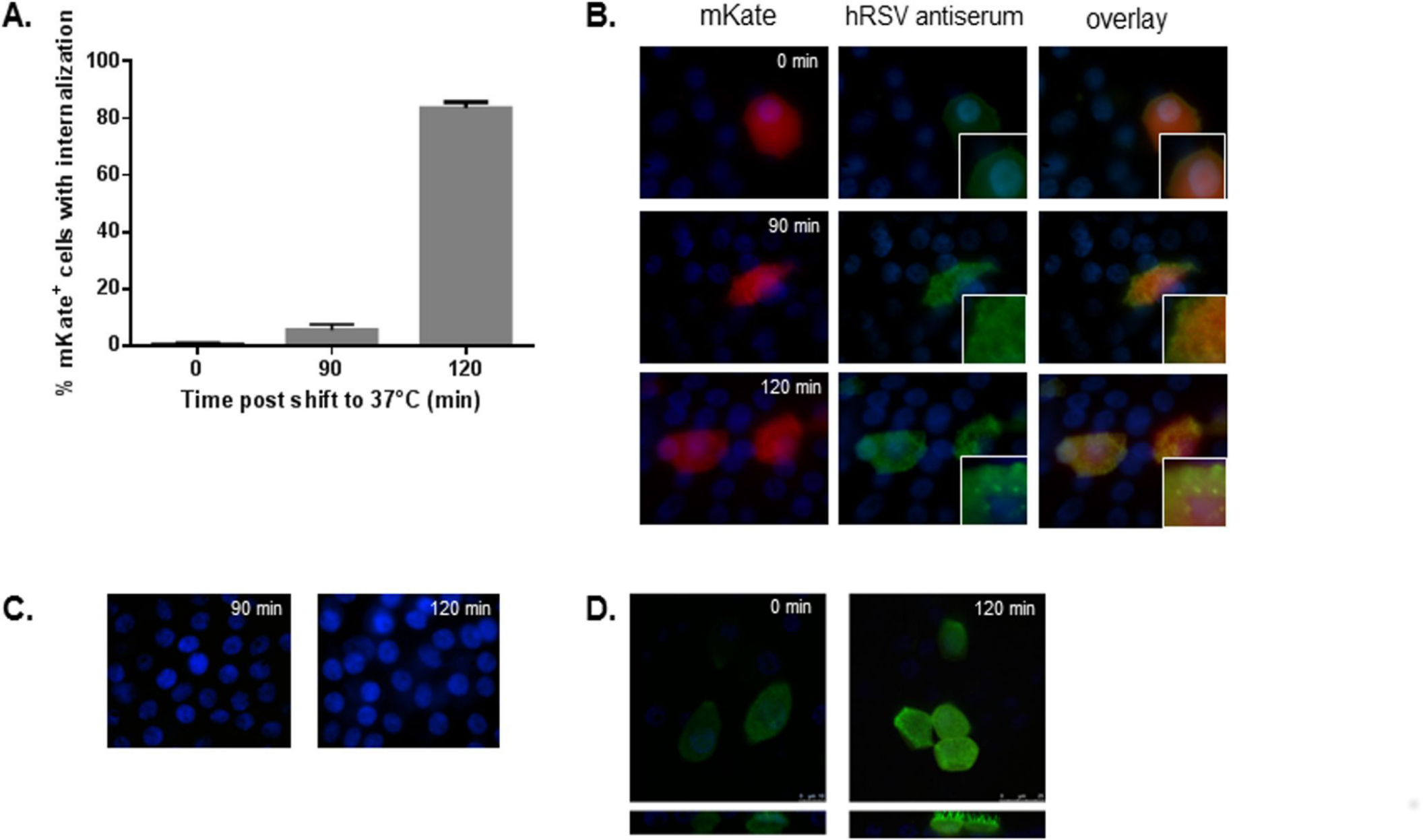
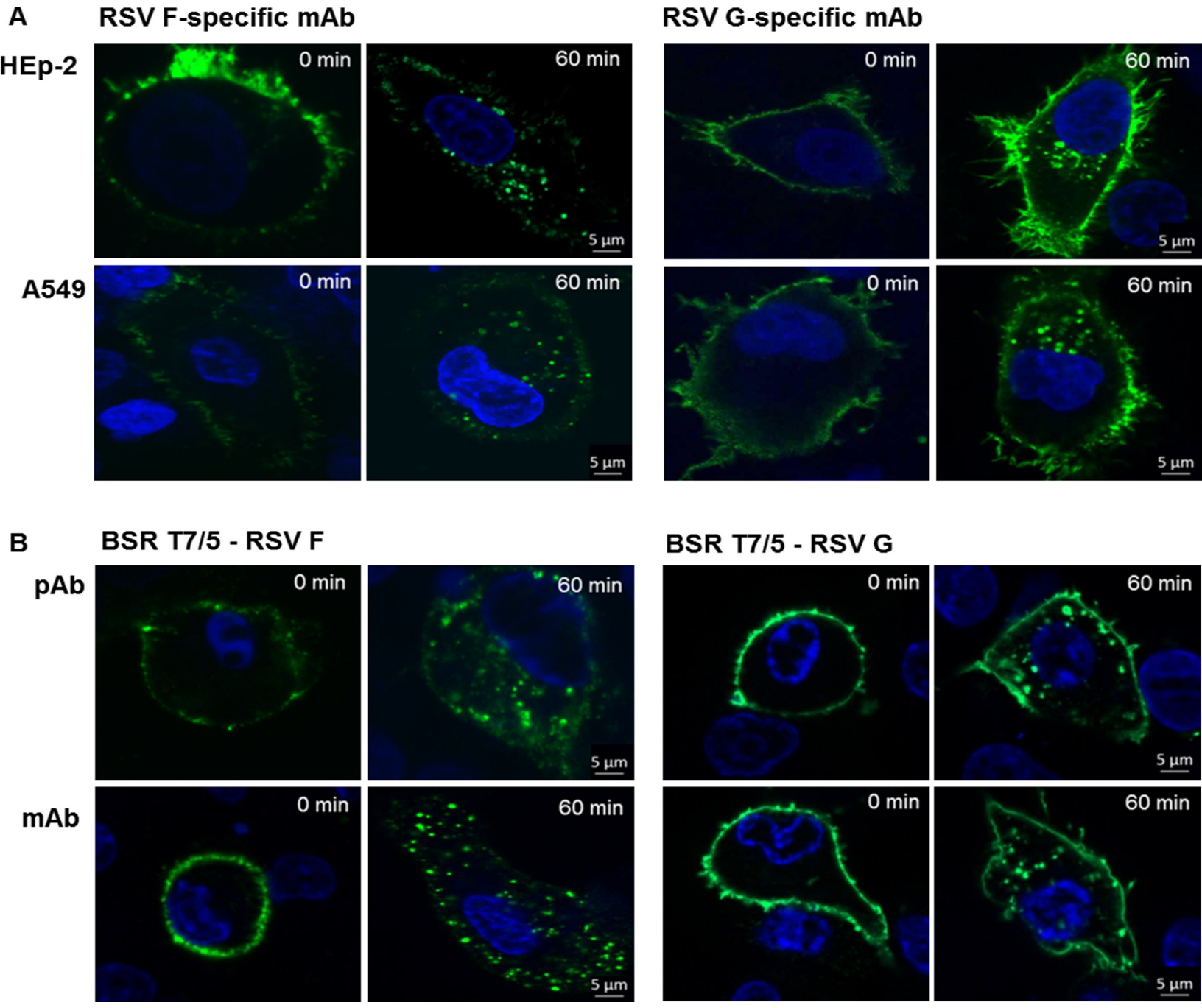
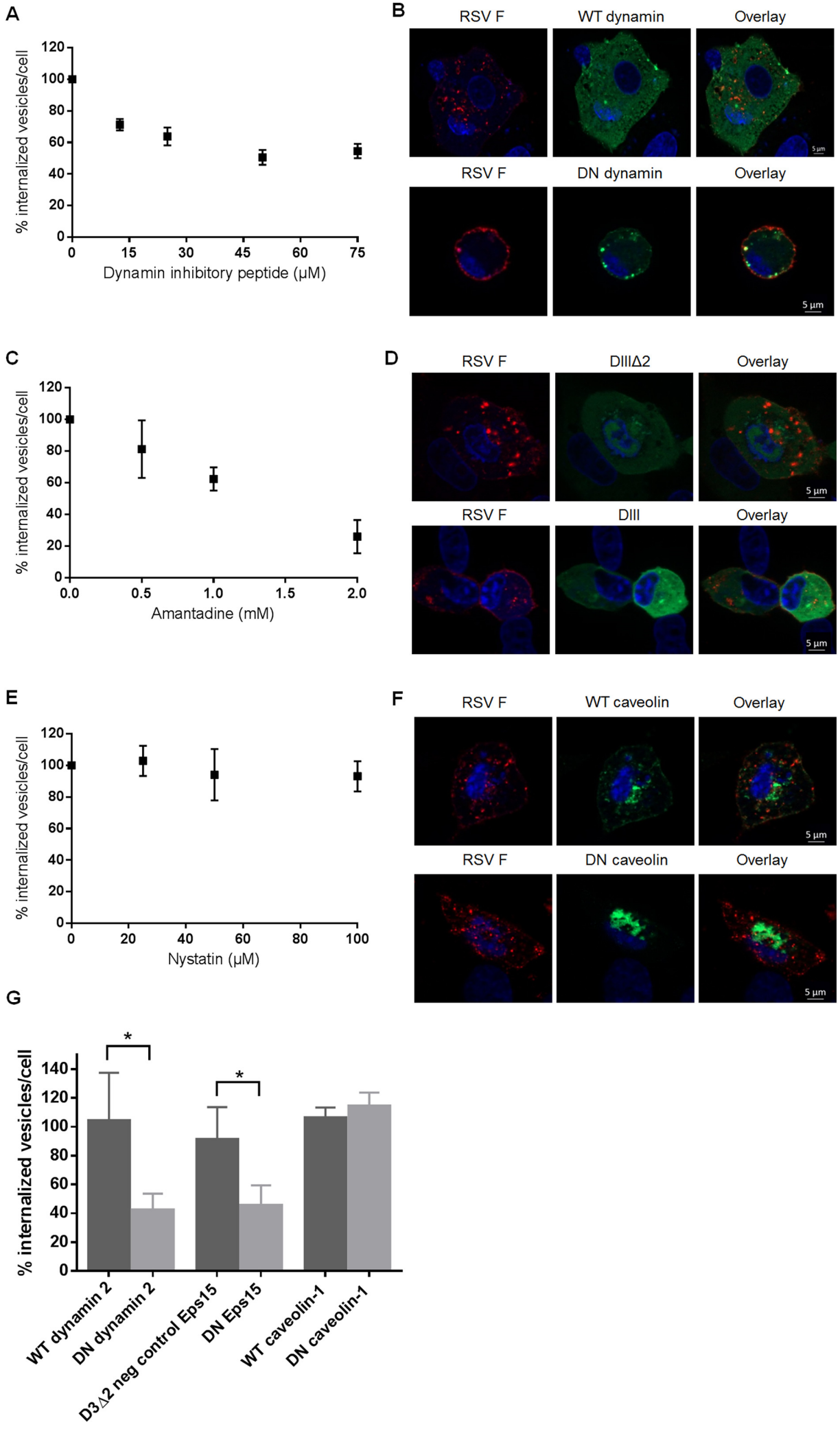
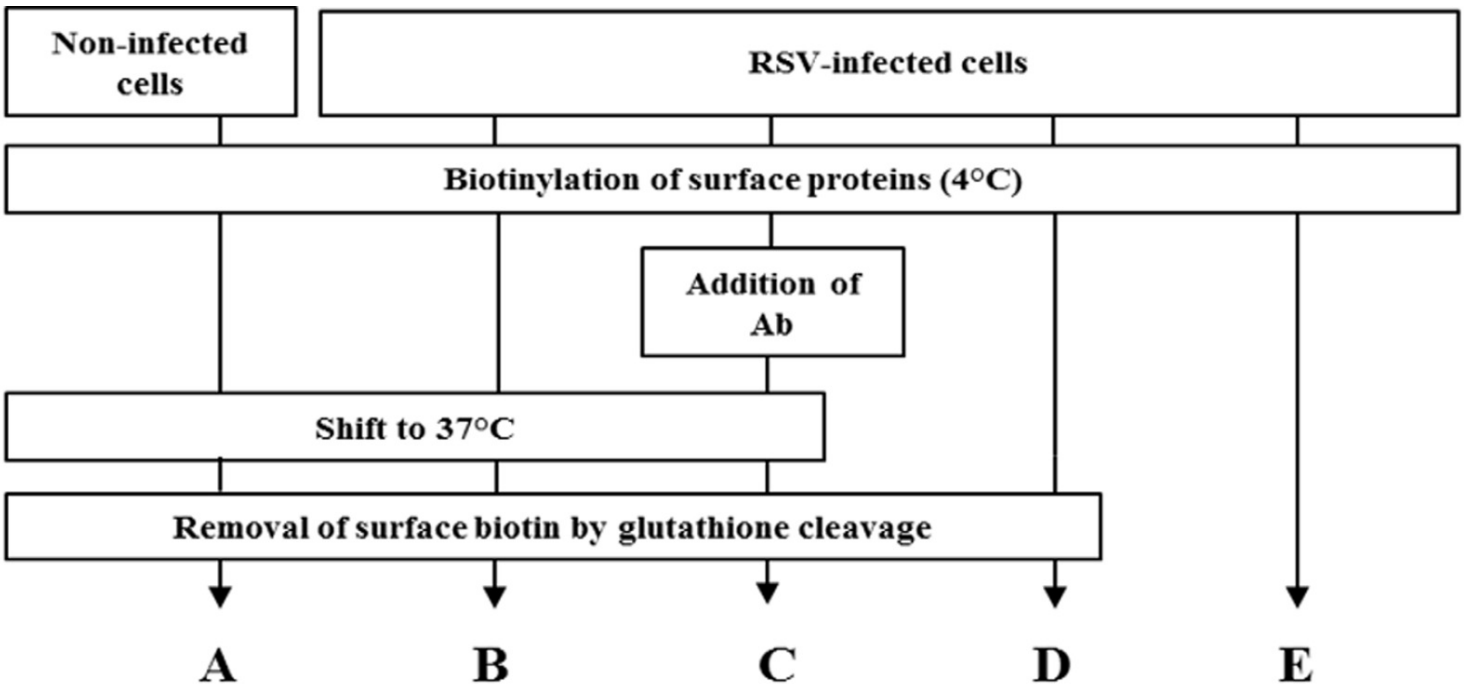
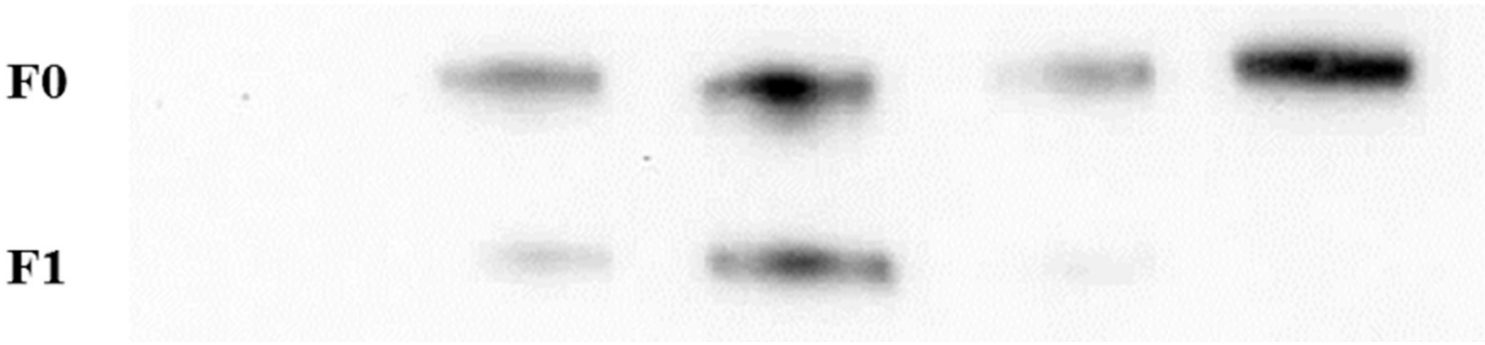
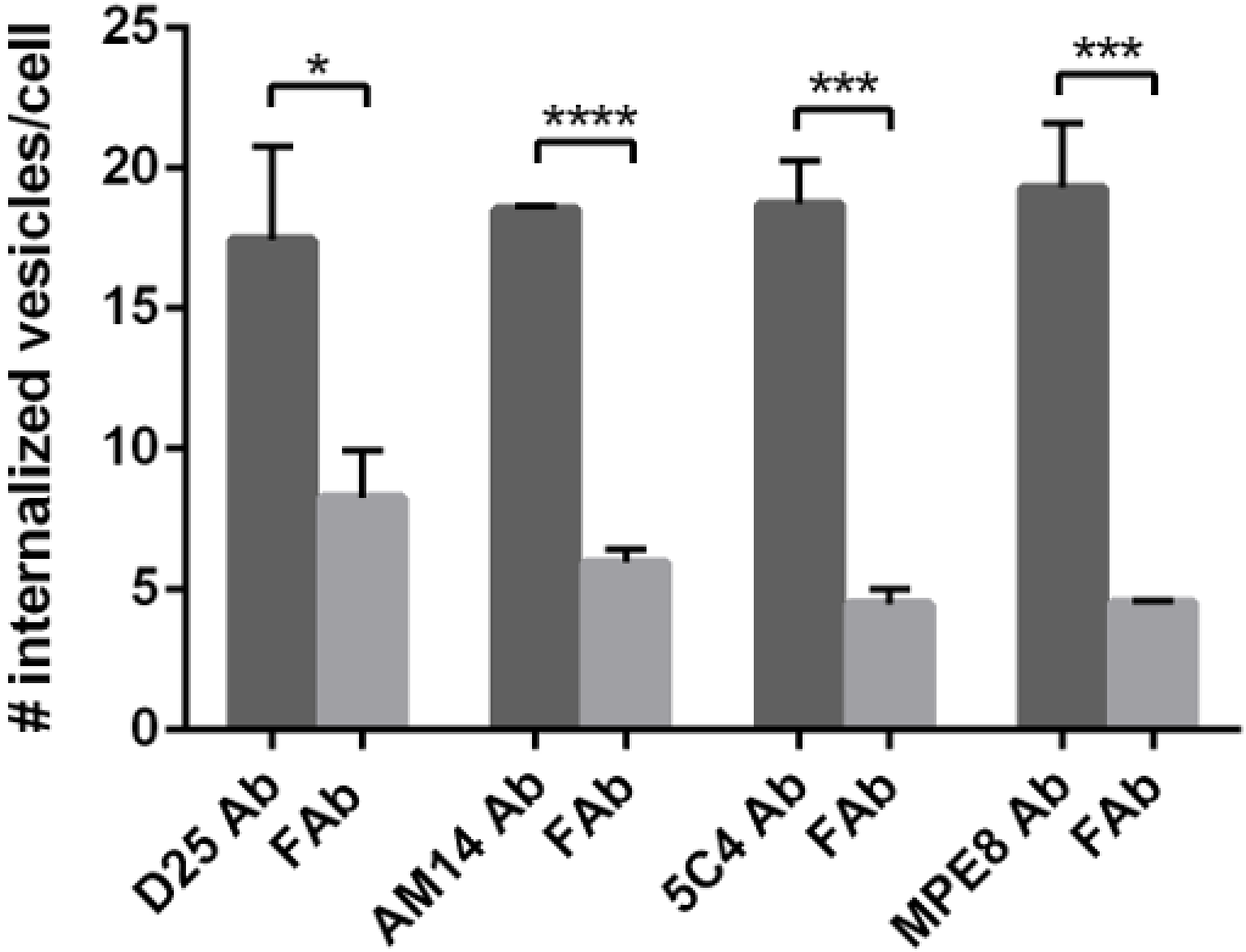

A.
% mKate+ cells with internalization
100
80
60
40
20
0
0
90
120
Time post shift to 37°C (min)
B.
mKate
hRSV antiserum
overlay
0 min
90 min
120 min
C.
90 min
120 min
D.
0 min
120 min


A
RSV F-specific mAb
RSV G-specific mAb
HEp-2
A549
0 min
60 min
5 µm
B
BSR T7/5 - RSV F
BSR T7/5 - RSV G
pAb
mAb


A
% internalized vesicles/cell
120
100
80
60
40
20
0
0
15
30
45
60
75
Dynamin inhibitory peptide (μM)
B
RSV F
WT dynamin
Overlay
5 μm
RSV F
DN dynamin
Overlay
5 μm
C
% internalized vesicles/cell
120
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Amantadine (mM)
D
RSV F
DIIIΔ2
Overlay
5 μm
RSV F
DIII
Overlay
5 μm
E
% internalized vesicles/cell
120
100
80
60
40
20
0
0
20
40
60
80
100
Nystatin (μM)
F
RSV F
WT caveolin
Overlay
5 μm
RSV F
DN caveolin
Overlay
5 μm
G
% internalized vesicles/cell
140
120
100
80
60
40
20
0
*
*
WT dynamin 2
DN dynamin 2
D3Δ2 neg control Eps15
DN Eps15
WT caveolin-1
DN caveolin-1


Non-infected cells
RSV-infected cells
Biotinylation of surface proteins (4°C)
Addition of Ab
Shift to 37°C
Removal of surface biotin by glutathione cleavage
A
B
C
D
E


F0
F1


internalized vesicles/cell
25
20
15
10
5
0
*
****
***
***
D25 Ab
FAb
AM14 Ab
FAb
5C4 Ab
FAb
MPE8 Ab
FAb