Currently, the spatial distribution of human respiratory syncytial virus (hRSV) proteins and RNAs in infected cells is still under investigation, with many unanswered questions regarding the interaction of virus-induced structures and the innate immune system. Very few studies of hRSV have used subcellular imaging as a means to explore the changes in localization of retinoic-acid-inducible gene-I (RIG-I)-like receptors or the mitochondrial antiviral signaling (MAVS) protein, in response to the infection and formation of viral structures. In this investigation, we found that both RIG-I and melanoma differentiation-associated gene 5 (MDA5), both RNA helicases, and the pathogen-associated molecular pattern receptors, known as the Toll-like receptors (TLRs), respectively, involve two kinds of receptors: the cytoplasmic pattern recognition receptors, including the retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), both RNA helicases, and the pathogen-associated molecular pattern receptors, known as the Toll-like receptors (TLRs) (1, 18). TLR3 and TLR7 sense double- and single-stranded RNA, respectively, and in most cell types reside within endosomes (18). TLR signaling, mediated by MyD88, and RIG-I-like receptor signaling, mediated by the mitochondrial antiviral signaling (MAVS) protein (also known as the interferon promoter-stimulating factor-1 [IPS-1] adaptor protein), results in the production of interferon (18).

In response to hRSV, both TLRs and RIG-I-like receptors have been implicated in the general response to the infection. The hRSV fusion (F) protein has been shown to antagonize TLR4 in a mouse model (12). In A549 and mouse embryonic fibroblast (MEF) cells, it has been shown that RIG-I was essential for promoting the innate immune response, with MDA5 playing an auxiliary role (16, 17, 30). Even though there has been some confusion in the literature, recent results depicting the early events (0 to 6 h postinfection [hpi]) of an hRSV infection have shown that both RIG-I and MDA5 play a significant role in both NF-κB and IRF3 signaling (30).

In addition, a number of viral proteins have been implicated in decreasing the innate response to hRSV. The highly glycosylated attachment (G) protein has been shown to inhibit TLR3/4 signaling in dendritic cells. Nonstructural protein 1 (NS1) expression has been correlated with a decrease in TRAF3 levels, and nonstructural protein 2 (NS2) has been shown to interact with RIG-I and its expression to correlate with decreases in TRAF3 and STAT2 levels (2, 27).

One drawback of many investigations of hRSV induction of the innate immune response to date has been the lack of spatiotemporal information at the level of the single cell. Previous work in this area has provided considerable insight primarily through biochemical assays, but these investigations often have neglected to study the events of interest within the context of the cell, retaining the spatial organization of hRSV proteins and RNAs. Many representations of the signaling events during hRSV infections depict hRSV genomic viral RNA as randomly distributed throughout the cytoplasm even though this pattern has never been shown via microscopic investigation. Recent time course imaging of hRSV genomic RNA using single-molecule sensitive hybridization probes clearly showed the RNA and the N protein localized near...
the plasma membrane in granules of various size during the first 6 h of infection in HEp-2 cells. The pattern progressed to a mixture of smaller granules near the cell membrane and moderate to large inclusion bodies, both at the cell membrane and deeper within the cells, at 12 h postinoculation (14). By 24 hpi, the plasma membrane contained many small granules, filaments, and inclusion bodies, as well as inclusions deeper within the cell. It was observed via subsequent immunofluorescence staining that the N protein colocalized frequently (Manders overlap coefficient, >60%) with the genomic RNA in smaller granules (on the order of the point spread function, ~250 nm) but less frequently in moderate to large inclusion bodies, which often stained around their exterior with anti-N antibodies.

Currently, the function of inclusion bodies in hRSV-infected cells is unknown. It has been speculated that they are sites of replication or transcription, morphogenic intermediates, or sites for the accumulation of dead-end products. Since their earliest mention in the literature, cytoplasmic inclusion bodies have been observed late in hRSV infections using both light and electron microscopy, both in cells infected in culture and in cells from infected children or animals. Inclusions of various size have been immunostained with antibodies to the N, P, M, M2-1, and L proteins, but not for the F or G proteins (5–9), and viral genomic RNA also has been detected either within them or around their perimeter, depending on their size (14, 22). Inclusion bodies have been shown to form spontaneously within cells in culture following the expression of both the N and P proteins, forming inclusions morphologically similar to those observed during viral infections (6). When N or P was expressed separately, inclusions did not form; instead the proteins were distributed evenly throughout the cytoplasm.

Given our previous observations of hRSV organization, our initial goal in the current studies was to couple our previous localization results with additional information regarding the localization of components of the innate immune response such as RIG-I, MDA5, and MAVS, during an hRSV infection. The experiments yielded a number of interesting findings, including data showing that RIG-I and MDA5 colocalized with hRSV granules in the cytoplasm near the plasma membrane as soon as 6 hpi, with MAVS remaining localized to mitochondria. By 12 hpi, MAVS was still partially localized to the mitochondria, but also clearly localized within inclusion bodies, as was MDA5. By 24 hpi, the detectable fraction of MAVS and MDA5 were contained within hRSV granules and inclusion bodies. Given that granules and inclusions can be formed from the coexpression of N and P, we investigated whether the changes in localization observed during the infection occurred with the expression of N and P in the absence of other viral components, and found that the changes in localization still occurred. Next, we postulated that N was interacting with MAVS and MDA5, so we developed a proximity ligation assay (PLA) to assess this interaction in situ. PLAs use oligonucleotide-linked antibodies, distance-dependent ligation of DNA to induce circularization, rolling circle amplification, and fluorescently labeled DNA hybridization to test if two proteins interact. The assays can exhibit single interaction sensitivity within a distance of 40 nm (25, 26). Employing such assays we confirmed that N, either expressed alone or simultaneously with the P protein, was in proximity to MAVS and MDA5 in situ. It was also found that hRSV N coprecipitated with MDA5 when overexpressed, indicating an interaction, direct or indirect, between the two proteins. Further, in order to confirm the function of the sequestration of these proteins within inclusion bodies, N and P were coexpressed, and the cells were subsequently infected with Newcastle disease virus (NDV) (LaSota strain). These experiments showed a significant decrease in interferon β mRNA production compared to that during the wild-type infection, giving credence to the function of inclusion bodies as inhibitors of the innate immune response.

Overall, through an examination of the spatiotemporal changes in the localization of proteins involved in the innate immune response, we identified for the first time that the hRSV N protein and subsequent inclusion body formation serve to antagonize the innate immune response likely by interacting with and localizing MDA5 and MAVS.

MATERIALS AND METHODS

MTRIPs. Multiply labeled tetravalent imaging probes (MTRIPs) were prepared as previously described (23). Briefly, 2′-O-methyl RNA/DNA
FIG 2 hRSV IBs colocalize with MAVS and RIG-I. A549 cells were infected with RSV strain A2 at an MOI of 1.0 and incubated for 6, 12, or 24 h or were mock infected. hRSV N, MAVS, and RIG-I were detected by immunofluorescence. Column 1, RSV N staining; column 2, MAVS staining; column 3, RIG-I staining. Column 4, merge with hRSV N colored green, MAVS colored red, and RIG-I colored blue. The nucleus, stained by DAPI, is colored white. Column 5, increased-magnification view of the boxes in the merged images. Below each panel is an intensity line graph showing the intensity of each channel along the line in the cropped image. Detail of filaments in a higher optical plane of the cell at the 24-hpi time point is shown in row 5. Scale bars, 10 μm (column 4, rows 1 to 4) and 5 μm (column 5 and row 5). Images for the mock infections are wide-field only due to a computational artifact of deconvolving background without positive signal.
chimeric oligonucleotides were synthesized by Biosearch Technologies, Inc., with the following sequence: 5'-biotin-UGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUG
hRSV IBs colocalize with MDA5 and RIG-I. A549 cells were infected with hRSV strain A2 at an MOI of 1.0 and incubated for 6, 12, or 24 h or were mock infected. hRSV N, MDA5, and RIG-I were detected by immunofluorescence. Column 1, hRSV staining; column 2, MDA5 staining; column 3, RIG-I staining. Column 4, merge with hRSV N colored green, MDA5 colored red, and RIG-I colored blue. The nucleus, stained by DAPI, is colored white. Column 5, increased-magnification view of the boxes in the merge image. Below each panel is an intensity line graph showing the intensity of each channel along the line in the cropped image. Detail of filaments in a higher optical plane of the cell at the 24-hpi time point is shown in row 5. The arrows in row 5 point to an inclusion body (upper) and a viral filament (lower). Scale bars, 10 μm (column 4, rows 1 to 4) and 5 μm (column 5 and row 5). Images for the mock infections are wide-field only due to a computational artifact of deconvolving background without positive signal.
tease inhibitor (Roche). Lysates were stored at −80°C until use. For immunoprecipitation, Dynabeads coated with sheep anti-rabbit antibodies (Invitrogen) were incubated overnight at 4°C with rabbit anti-GFP antibodies (Thermo). The beads were washed, and lysates were added to the beads. Lysates were tumbled overnight at 4°C. The beads were washed to remove unbound proteins. To release the precipitate, SDS buffer (LI-COR Biosciences) was added to the beads and the solution was incubated at 70°C for 10 min. The eluted proteins were loaded into 10% Bis-Tris gels (Invitrogen) and run using MOPS (morpholinepropanesulfonic acid) buffer (Invitrogen). Western transfer was performed in an XCell II blot module (Invitrogen) onto nitrocellulose paper (Invitrogen) according to the manufacturer’s protocol. Blotting was performed using a Snap i.d. (Millipore). Blots were blocked using Odyssey blocking buffer (LI-COR Biosciences), and primary antibodies, mouse anti-GFP and mouse anti-RSV N, were diluted in Odyssey blocking buffer with 0.1% Tween 20 (CalBioChem) and incubated on the blots for 10 min. Blots were washed in PBS with 0.1% Tween 20. Secondary goat anti-mouse antibody labeled with DyLight 680B (Thermo), diluted as described above, was incubated for 10 min. Blots were washed as described above. Blots were imaged on an Odyssey (LI-COR Biosciences) at 42 m pixel⁻¹ using the “high” quality setting. Blots were linearly contrast enhanced in ImageJ for clarity. Bands corresponding to RSV N and the GFP-MAVS were differentially enhanced due to the sensitivity differences of the primary antibodies against the respective proteins.

RESULTS
Localization and structure of granules associated with hRSV N protein and vRNA during infection. We first analyzed the structures and distribution of viral proteins and genomic RNA at 6, 12, or 24 h postinfection (hpi). HEp-2 cell monolayers were inoculated with hRSV strain A2 at an MOI of 1 and incubated in growth medium for the indicated times. Multiply labeled tetravalent imaging probes (MTRIPs) were delivered into the live cells as described, and they were subsequently fixed and immunostained for hRSV N (Fig. 1). At 6 hpi, both the viral genomic RNA and inclusion bodies (IBs) positive for hRSV N protein were punctate and colocalized (Fig. 1, row 1). Morphologically, the IBs were round, and no filaments were present. The average volume of the IBs at this time point was 0.9 μm³. At 12 hpi, hRSV N staining was seen both as a diffuse population that filled the cytoplasm and as inclusion bodies, which were larger than those detected at 6 hpi (Fig. 1, row 2). These larger inclusion bodies (>8 μm³, with an average size of 37 μm³) did not contain genomic RNA detectable with probes, unlike those at 6 hpi. The genomic RNA was located predominantly at the plasma membrane and in smaller granules within the cytoplasm. At 24 hpi, N protein could also be detected in viral filaments on the plasma membrane of the cells in addition to the diffuse hRSV N staining and the staining of IBs (Fig. 1, rows 3 to 5). These filaments contained viral genomic RNA, as previously described (14, 22, 29). Notably the larger IBs contained little to no genomic RNA despite containing high levels of the N protein, which binds to the genomic RNA in the virion.

Viral inclusion bodies colocalize with proteins of the innate immune response. To test whether the hRSV IBs contained proteins of the RIG-I-like receptor (RLR) antiviral response pathway, we infected A549 cells and fixed them at 6, 12, or 24 hpi, or prior to infection (mock), and stained them for hRSV N, MAVS, and RIG-I. In the mock-infected cells no RSV N or RIG-I staining was present and MAVS staining appeared to be mitochondrial (Fig. 2, row 1). At 6 hpi, hRSV N staining was seen both as a diffuse population that filled the cytoplasm and as inclusion bodies, which were larger than those detected at 6 hpi (Fig. 1, row 2). These larger inclusion bodies (>8 μm³, with an average size of 37 μm³) did not contain genomic RNA detectable with probes, unlike those at 6 hpi. The genomic RNA was located predominantly at the plasma membrane and in smaller granules within the cytoplasm. At 24 hpi, N protein could also be detected in viral filaments on the plasma membrane of the cells in addition to the diffuse hRSV N staining and the staining of IBs (Fig. 1, rows 3 to 5). These filaments contained viral genomic RNA, as previously described (14, 22, 29). Notably the larger IBs contained little to no genomic RNA despite containing high levels of the N protein, which binds to the genomic RNA in the virion.

MDA5 colocalized with isolated viral filaments. HEp-2 cells were infected with hRSV strain A2 at an MOI of 0.1, and viral filaments were isolated 4 dpi. Viral filaments were isolated by filtration and adsorbed onto cover glass for immunostaining. Column 1, host protein RIG-I, MDA5, or MAVS, red in the merge. Column 2, hRSV N staining, green in the merge. Column 3, hRSV F staining, blue in the merge. Column 4, merge. Column 5, intensity profile through the lines in the merged images; arrow, pixel number 1. Scale bar, 1 μm.

FIG 4 MDA5 colocalized with isolated viral filaments. HEp-2 cells were infected with hRSV strain A2 at an MOI of 0.1, and viral filaments were isolated 4 dpi. Viral filaments were isolated by filtration and adsorbed onto cover glass for immunostaining. Column 1, host protein RIG-I, MDA5, or MAVS, red in the merge. Column 2, hRSV N staining, green in the merge. Column 3, hRSV F staining, blue in the merge. Column 4, merge. Column 5, intensity profile through the lines in the merged images; arrow, pixel number 1. Scale bar, 1 μm.
plasm, indicative of mitochondria, as would be expected (Fig. 2, row 2) (24). RIG-I staining was similar to hRSV N staining and was punctate. All three proteins were present inside the hRSV N IBs, with a strong correlation between the hRSV N staining and the RIG-I staining. At 12 hpi, hRSV N was again in larger IBs that appeared to be hollow (Fig. 2, row 3). MAVS staining at 12 hpi was altered dramatically, with some of the protein localizing to large IBs that were not observed at earlier time points and appeared to be surrounded by a “ring” of hRSV N staining. Cytoplasmic MAVS staining was still present; however, this staining was now longer filamentous and did not resemble mitochondria. RIG-I staining also was increased inside the larger IBs as well as throughout the cytoplasm. At 24 hpi, both MAVS and RIG-I were localized to large IBs and to viral filaments at the plasma membrane (Fig. 2, rows 4 and 5).

In order to see if this change in protein localization was specific to RIG-I/MAVS or was a more general phenomena of the RLR pathway, we next costained for hRSV N, RIG-I, and MDA-5. In the mock-infected cells no RSV N, MDA5, or RIG-I staining was present (Fig. 3, row 1). At 6 hpi, MDA5 was predominately coincident with IBs containing hRSV N, with little cytoplasmic staining apparent outside these regions (Fig. 3, row 2). At 12 hpi, MDA5 was predominately coincident with IBs containing hRSV N, with little cytoplasmic staining apparent outside these regions (Fig. 3, row 2). At 24 hpi, MDA5 colocalized with IBs of all sizes, and there was little to no staining detected outside these regions (Fig. 3, row 3). At 24 hpi, MDA5, like RIG-I, was present in viral filaments at the plasma membrane (Fig. 3, rows 4 and 5).
Owing to the dense nature of viral and host proteins near the plasma membrane, it was difficult to separate the bright signal from IBs from the lower signal inside filaments. To circumvent this problem, viral filaments were isolated from infected cells, deposited and fixed on glass coverslips, and immunostained for hRSV F and hRSV N, and RIG-I, MDA5, or MAVS (Fig. 4). While MDA5 stained strongly in the isolated filaments, neither RIG-I nor MAVS was observed to colocalize with them.

Expression of hRSV N and P proteins was sufficient to localize proteins of the innate immune response. In order to test whether the presence of IBs alone was sufficient to localize RIG-I, MDA5, and MAVS, hRSV N and P were expressed in Vero cells that subsequently were fixed and immunostained. As seen in the left column of Fig. 5, the expression of hRSV N and P resulted in the formation of both small and large IB-like structures similar to those seen in the infection model. In these cells, RIG-I staining was increased in and around the IBs; however, RIG-I was not localized exclusively to the IBs. In contrast, both MDA5 and MAVS were localized almost exclusively to the IBs, and little staining was detected outside these regions. In order to quantify these results, colocalization data were obtained from over 100 cells from five fields and were summarized in the graph in Fig. 5. A colocalization analysis between N and RIG-I yielded a Pearson’s coefficient of 0.5 and a Manders overlap of 0.4, suggesting the colocalization was strong but not complete. When the Manders overlap was broken into its constitutive parts, Mx and My, it was seen that three-quarters of the N staining colocalized with RIG-I (My, ∼0.75), while RIG-I staining colocalized with N approximately 25% of the time (Mx, ∼0.25). In contrast, the Pearson coefficient and Manders overlap for N with MDA5 or MAVS were both >0.75, and the Mx and My colocalization coefficients in these two cases were both >0.5, indicating near-complete colocalization between the expressed inclusion bodies and MDA5 and MAVS.

To verify that neither the antibodies were cross-reacting, nor the fluorophores bleeding through to adjacent channels, cells infected with RSV for 24 h were stained with a single primary antibody and all three secondary antibodies, anti-mouse Alexa Fluor 488, donkey anti-rabbit Cy3, and donkey anti-goat Cy5 (Fig. 6). Signal was present only in the correct channel for the primary-secondary antibody combination, indicating that bleed-through of signal from particularly intense staining, such as RSV N, was not occurring at the exposure times used, nor was the cross-reactivity of the secondary antibodies leading to detectable signal. There was more background in the Cy5 channel; however, the background was not due to bleed-through or cross-reaction, as both of those would lead to the appearance of IBs in the Cy5 channel coincident with those in the Cy3 or 488 channels, and this result was not observed.

**hRSV N is in close proximity to MDA5 and MAVS in situ.** The colocalization analysis discussed above suggested an interaction between the proteins of the innate immune response and the viral inclusion bodies. However, owing to the limited spatial resolution of the light microscope (∼250 nm), a separate assay was needed to demonstrate that the proteins were in close proximity (<30 nm) and likely interacting in the cell. Immunoprecipitation is commonly used to demonstrate that two or more proteins interact; however, it is fundamentally an *in vitro* assay. The localization of interactions *in situ* is lost and rearrangements of proteins postlysis may interfere with the interpretation of the results. In order to avoid these complications, we employed a proximity ligation assay (PLA) to demonstrate the close proximity of N with MAVS and MDA5 *in situ* during viral infection. Figure 7 shows Vero cells stained for hRSV P (to identify hRSV IBs) and PLA between N and MAVS or MDA5 at 6 and 24 hpi. PLA control experiments can be seen in Fig. 8. At 6 hpi, hRSV P staining resembled the IB granules marked by hRSV N, and the PLA signal between N and MDA5 had
a nearly one-to-one correspondence with the signal from the hRSV P. At 24 hpi, hRSV P staining showed both IB and filamentous structures. The PLA signal between hRSV N and MDA5 at this time point was significantly stronger and, while it is much more heterogeneous than at 6 hpi, there was still a strong correlation between the IBs and filaments with the PLA signal. When PLA was performed between hRSV N and MAVS, there were fewer PLA granules observed. At 24 hpi, this increased; however, it never reached the levels of that of hRSV N/MDA5, and the colocalization between the PLA and hRSV P staining was lower.

hRSV N interacts with MDA5 and MAVS. Vero cells were infected with hRSV strain A2 at an MOI of 1.0 and incubated for 6 or 24 h. Cells were stained for hRSV P and were assayed for the interaction between hRSV N and either MDA5 or MAVS by PLA. Column 1, immunostaining for hRSV P. Column 2, PLA signal from either hRSV N-MDA5 assays (rows 1 and 2) or hRSV N-MAVS assays (rows 3 and 4). Column 3, merge of column 1 (green) and column 2 (red), with the nucleus stained with DAPI in blue. Column 4, magnification of the boxed region in the panels in column 3. The bar graph shows the mean number of PLA spots per cell detected for each experimental condition along with those for the negative controls (Fig. 8). Error bars are standard deviations. Scale bars, 10 μm (column 3) and 5 μm (column 4).

hRSV N is in close proximity to MAVS and MDA5 but not RIG-I in the absence of viral infection. To test whether viral replication was required for the interaction between hRSV N and the proteins of the RLR response, PLA was performed between hRSV N-GFP and hRSV P or only hRSV N-GFP (Fig. 9 and controls in Fig. 10). When hRSV N-GFP was expressed alone, a PLA signal was observed with MDA5 and MAVS, indicating that neither an active viral infection nor any other components of the virus were necessary for this reaction (Fig. 9, rows 2 and 3). In contrast, when PLA was performed between hRSV N and RIG-I, no PLA signal...
was observed, indicating that the two were not in close proximity (Fig. 9, row 1). When hRSV N-GFP and hRSV P were coexpressed, the PLA signal was still observed between N and MAVS or MDA5; however, its localization had changed. Instead of being diffuse and cytoplasmic as in the case when hRSV N-GFP was expressed alone, the signal was predominantly around IBs when hRSV N-GFP and hRSV P were coexpressed (Fig. 9, rows 5 and 6). Interestingly, even though RIG-I was present in inclusion bodies when RSV N and P were coexpressed (Fig. 4), it was not in close enough proximity to give a signal from the PLA (Fig. 9, row 4). While MDA5 is soluble and available to bind to targets throughout the cytoplasm, MAVS is localized predominantly to the mitochondria. How MAVS translocated from the mitochondrial surface to IBs is unknown. However, when hRSV N-GFP was coexpressed with the hRSV P and the mitochondria were labeled with MitoTracker, we observed that the mitochondria often surrounded the IBs (Fig. 11). Figure 11A shows a living cell in which the IBs formed from N and P expression were surrounded by mitochondria. To better observe this phenomenon, a 3-D isosurface view was generated (Fig. 11C) from the field in Fig. 11B.

To confirm that RSV N either directly or indirectly interacted as part of a larger complex with MDA5 and MAVS, both GFP-MAVS and GFP-MDA5 were coexpressed with RSV N and were immunoprecipitated using an anti-GFP antibody (Fig. 12). When the precipitates were subjected to Western blotting it was found that N was present in the GFP-MAVS precipitate but not in the GFP-MAVS fraction (Fig. 12, lanes 1 and 2). No primary antibody controls were negative for the GFP-MAVS, GFP-MAVS, and RSV N proteins (Fig. 12, lanes 3 and 4).

RSV P is in proximity to MDA5 only when coexpressed with RSV N. To test if the proximity of RSV N-GFP and MDA5 and MAVS is unique to RSV N, RSV P-GFP was expressed alone or in combination with RSV N and a PLA was performed between RSV P and MAVS or MDA5. When RSV P-GFP was expressed alone, no PLA signal was observed with either MAVS or MDA5, indicating that overexpression of the viral protein is not sufficient to bring the two into close proximity. When coexpressed with RSV N, PLA signal was observed between RSV P-GFP and MDA5 but not MAVS, indicating that RSV P forms a complex with RSV N that brings it sufficiently close (<30 nm) to MDA5 but not MAVS.

Notably, PLA between RSV P-GFP and MDA5 was exclusively observed around the periphery of IBs. This is in contrast to the PLA between RSV N-GFP and MDA5 or MAVS, which was predominantly but not exclusively localized to IBs (Fig. 13).

To further test if the PLA between RSV N and cellular proteins is an artifact of overexpression of the viral protein, a PLA was performed between RSV N (without the GFP fusion) and a coexpressed GFP control plasmid, which expresses a cytoplasmic, diffusely localized GFP. No PLA signal was observed, indicating that even overexpression of both proteins is insufficient to give a PLA signal if they do not interact. A positive control, RSV N-GFP expression and intramolecular PLA between the RSV N and GFP domains, demonstrated a high level of PLA signal (Fig. 14).

Expression of hRSV N and coexpression of hRSV N and P resulted in the attenuation of interferon response. Given the data presented above, we hypothesized that the interaction of hRSV N with MAVS and MDA5 and the sequestration of these proteins into IBs during infection may cause a decrease in the expression of type 1 interferons and, specifically, interferon β. To test this hypothesis, we transfected HeLa cells with either a GFP control expression plasmid, hRSV N-GFP alone, or hRSV P-GFP alone or cotransfected them with hRSV N-GFP and P. The cells

FIG 8 PLA gave no signal in primary antibody control cells infected with RSV. Vero cells were infected with hRSV strain A2 at an MOI of 1.0 and incubated for 6 or 24 h. Cells were stained for RSV P (green) and were assayed for the interaction between RSV N and either MDA5 or MAVS by PLA (red) without the primary antibody for hRSV N. The nucleus, stained by DAPI, is colored blue. Scale bars, 10 μm.

FIG 9 hRSV N is in proximity to MDA5 and MAVS but not RIG-I in the absence of viral infection. Vero cells were transfected with plasmids encoding either hRSV N-GFP or hRSV N-GFP and hRSV P and assayed for the interaction between hRSV N and RIG-I, MDA5, or MAVS. Column 1, GFP signal from the hRSV N-GFP. Column 2, PLA signal for hRSV N with RIG-I (rows 1 and 4), MAVS (rows 2 and 5), or hRSV N with MDA5 (rows 3 and 6). Column 3, merge of column 1 (green) with column 2 (red), with the nucleus stained with DAPI in blue. Column 4, magnification of the boxed region in the panels in column 3. Scale bars, 10 μm (column 3) and 5 μm (column 4).
were subsequently inoculated with the LaSota strain of Newcastle disease virus (NDV), a pathogen known to induce an interferon response through the RLR pathway in HeLa cells (31). At 24 h after NDV inoculation, total RNA was isolated from the cells, and beta interferon (IFNB1) mRNA was quantified relative to \( /H9252 \)-actin (ACTB) mRNA using reverse transcription real-time PCR (Fig. 15). Cells transfected with either hRSV N-GFP or hRSV N-GFP and P and infected with NDV expressed IFNB1 at a level 27 times lower than in cells transfected with GFP control plasmid and infected with NDV (Fig. 15B). When smaller amounts of hRSV N-GFP DNA were transfected into the cells, the effect of hRSV N on interferon expression was diminished in a dose-dependent fashion. In addition, the expression of P alone had no effect on IFNB1 expression, as suggested by the PLA data (Fig. 15B). These data indicated that the effect of hRSV N on the innate immune response lowers the production of IFNB1 when hRSV N is present as a diffuse population throughout the cytoplasm and when it is localized to cytoplasmic IBs.

In untransfected HeLa cells, NDV infection caused IFNB1 expression to increase 150,000-fold at 24 hpi relative to that in uninfected, untransfected HeLa cells. In cells that had been transfected with a GFP control plasmid, NDV infection caused IFNB1 expression to increase 5,000-fold when infected with NDV relative to that in uninfected HeLa cells transfected with GFP. The difference in IFNB1 expression may be due to the transfection process via Lipofectamine 2000. To test if this was the case, cells were treated with the transfection agent alone and infected with NDV. As can be seen in Fig. 15A, the transfection agent, without DNA, reduced the susceptibility of the cells to IFN induction when infected with NDV. While this reduces the overall effect of the sequestration of RLR components on the induction of IFNB1 by NDV in cells expressing hRSV N, there is still a sizable difference (~27-fold) compared to results for cells expressing GFP alone. In

![FIG 10 PLA gave no signal in primary antibody control cells transfected with plasmids encoding RSV N-GFP and RSV P, RSV N-GFP alone, RSV P-GFP and RSV N, or RSV P-GFP alone. Vero cells were transfected with plasmids encoding hRSV N-GFP, hRSV N-GFP and hRSV P, hRSV P-GFP, or hRSV P-GFP and hRSV N and assayed for the interaction between hRSV N and MAVS (column 1), MDA5 (column 2), or RIG-I (column 3) without the primary antibody for hRSV N.](image1)

![FIG 11 Mitochondria are found adjacent to hRSV IBs. Vero cells were transfected with plasmids encoding hRSV N-GFP and RSV P. At 24 h posttransfection, mitochondria were labeled with Mitotracker deep red 633 and imaged live. (A) Single cell. (B) Magnification of the boxed region in panel A. (C) Three-dimensional isosurface view of a large IB from panel B surrounded by mitochondria. hRSV N-GFP is colored green, Mitotracker is colored in red, and nuclei stained by DAPI are in blue. Scale bar, 10 \( /H9262 \) m (A) and 5 \( /H9262 \) m (B).](image2)

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![FIG 12 hRSV N coprecipitates with GFP-MDA5. HeLa cells were transfected with plasmids encoding GFP-MDA5 and hRSV N or GFP-MAVS and hRSV N. At 24 h posttransfection, cells were lysed and subjected to IP using an anti-GFP antibody or control antibody. Precipitates were subjected to electrophoresis and Western blotting using an anti-GFP to detect the GFP-MDA5 and GFP-MAVS and an anti-hRSV N antibody to detect the hRSV N. Blots were linearly contrast enhanced.](image3)
cells transfected with GFP alone, NDV infection caused IFNB1 expression to increase 27,000-fold relative to the amount in control HeLa cells (no transfection, no infection) and 5,000-fold relative to that in cells that were transfected with GFP and infected with NDV.

**DISCUSSION**

The experiments presented here demonstrate that one function of the large hRSV inclusion bodies might be to modulate the innate immune response to infection. Although previous experiments have implicated the nonstructural hRSV proteins NS1 and NS2 as modulators of the innate immune response, we demonstrated here that the N protein itself may be involved in that process (3, 4, 15). We studied the role of hRSV N and IBs in modulating the RLR-induced interferon response by colocalization analysis, proximity ligation assay, and immunoprecipitation and by a bioassay using an infection with Newcastle disease virus. We determined that RIG-I, MDA5, and MAVS colocalized with small and large hRSV IBs and that MDA5 and MAVS localized with hRSV N both inside IBs and when expressed diffusely within the cytoplasm. Furthermore, we demonstrated that hRSV N, both expressed diffusely and in IBs, significantly attenuated the interferon response induced by Newcastle disease virus infection in a dose-dependent fashion.

Cytoplasmic IBs, first observed by transmission electron microscopy (TEM), are likely aggregates of N proteins (19, 20). Despite the considerable body of knowledge in the molecular biology of hRSV, little is known about the composition, structure, and function of IBs. Previous studies have examined the viral content of IBs and the viral proteins necessary for their formation. It was found that expression of hRSV N and free GFP (N-OPT + GFP) or hRSV N-GFP alone (N-GFP). A PLA was performed against the GFP and hRSV N domains in both cases. PLA signal was quantified on a per-cell basis and summarized. Nuclei were stained with DAPI. ***, P < 0.001. N.D., not detected. GFP signal is shown in green, PLA signal is shown in red, and DAPI signal is shown in blue. Scale bars, 10 μm. Error bars are standard deviations.

**FIG 13** hRSV P is in proximity to MDA5 and MAVS in the absence of viral infection. Vero cells were transfected with plasmids encoding either hRSV P-GFP or hRSV P-GFP and hRSV N and assayed for the interaction between hRSV P and MDA5 or MAVS. Column 1, GFP signal from the hRSV P-GFP. Column 2, PLA signal for hRSV P with MDA5 (rows 1 and 3) or hRSV N with MDA5 (rows 2 and 4). Column 3, merge of column 1 (green) with column 2 (red) with the nucleus stained with DAPI in blue. Column 4, magnification of the boxed region in the panels in column 3. Scale bars, 10 μm (column 3) and 5 μm (column 4).

**FIG 14** PLA produces a signal in the presence of interacting antigens but not in diffusely localized, noninteracting antigens. Vero cells were transfected with plasmids encoding either hRSV N and free GFP (N-OPT + GFP) or hRSV N-GFP alone (N-GFP). A PLA was performed against the GFP and hRSV N domains in both cases. PLA signal was quantified on a per-cell basis and summarized. Nuclei were stained with DAPI. ***, P < 0.001. N.D., not detected. GFP signal is shown in green, PLA signal is shown in red, and DAPI signal is shown in blue. Scale bars, 10 μm. Error bars are standard deviations.
viral filaments during their formation later in the infection (~24 hpi). Contrastingly, even though larger IBs (>8 μm³) contain large quantities of N protein, which is known to complex with viral RNA to form nucleocapsids, they contain little if any genomic RNA, suggesting that this class of IB is likely not the site of viral replication.

By immunofluorescence microscopy, large IBs often are shown to have an open or hollow structure with stronger staining around the periphery of the IB (5, 6, 14). The reason for this structure is unknown, but it is likely an artifact of immunofluorescence, since expressed IBs generated by hRSV N-GFP and hRSV P appear solid by examination of the GFP signal (Fig. 16); when imaged by TEM, IBs appear solid and uniform (10, 19–21). Given that we have successfully immunostained for proteins within the larger IBs, it is unlikely that the hollow staining pattern is due solely to limited antibody diffusion in fixed IBs. Instead, we propose that this pattern is caused by epitope inaccessibility due to interactions with host proteins. The structure of the N protein–RNA complex of hRSV has been determined; however, the structure of IBs generated by the expression of hRSV N and P has not (28). The structure of IBs, examined in the future at a nanoscale level, may yield clues to the overall functionality of IBs.

Here we suggest that IBs localize proteins of the innate immune response in order to decrease downstream interferon signaling. We demonstrated in a model of hRSV infection that early in the infection, MAVS and MDA5 are localized to small IBs or granules that contain hRSV N and genomic RNA. At later time points, these innate immune molecules are localized to larger IBs that contain little if any genomic RNA and to viral filaments that also contain genomic viral RNA. In both of these cases, the colocalization between the cellular proteins and hRSV N was confirmed by proximity ligation assay (PLA). We further showed that both the colocalization in large IBs and the PLA results were independent of viral infection by expressing hRSV N and P in trans, and therefore these findings were not a result of MDA5 interaction with the viral RNA. Finally, we demonstrated, by immunoprecipitation, that hRSV N either directly or indirectly interacted with MDA5. Overall, the picture generated by our data suggests that N interacts with both MDA5 and P within a macromolecular complex. It also suggests that MAVS is a part of this complex, in close proximity to N, but not within 30 to 40 nm of P.

The innate immune response to RNA virus infections proceeds through two pathways, via RIG-I-like helicases, including RIG-I and MDA5, and the TLR pathway. Both of these pathways result in the induction of interferon production. The RIG-I-like proteins require an intermediary protein MAVS (also known as IPS-1, VISA, and Cardif), which binds to RIG-I-like helicases through a caspase activation and recruitment domain (CARD domain). MAVS initiates a signaling cascade by activating cytosolic kinases IKK and TBK1, which then activates transcription factors NF-κB and IRF3. These transcription factors translocate to the nucleus, where they promote the expression of type I interferons. In hRSV, the interferon response has been shown to play an important role in the nature and severity of the disease (11). MDA5 and RIG-I have both been found to be upregulated in infants infected with hRSV compared to infants that have not been infected.

The finding that MDA5 is efficiently localized into IBs may explain why RIG-I has been shown to have a larger effect on the
innate immune response to hRSV infection (17). The data also suggest a mechanism by which cells can be potentiated toward subsequent infection or coinfection. We have shown that the expression of IBs by hRSV localizes pattern recognition receptors of the RLR pathway and additionally localized their downstream effector MAVS. We demonstrated that N and IBs alone, and not P alone, are enough to diminish the cell’s ability to produce interferon in response to a viral infection. To our knowledge, this is the first report of hRSV IBs having a functional role in the life cycle of the virus. It is important to note that while cytoplasmic, diffuse hRSV N expression can decrease the interferon response without the formation of the inclusion bodies, in vivo hRSV N is highly localized to IBs (13).

In summary, there are few previous data describing the host cell protein content inside IBs or their precise function. The study presented here suggests an interaction of hRSV N protein with important host proteins of the innate immune response and a specific function for inclusion bodies. The findings also clearly point to the need for further studies regarding IB formation, structure, and function in terms of time and space during hRSV infections.

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