

Hepatitis B Immunoglobulin Inhibits The Secretion of HBV Via Antigen-Antibody Precipitation in The Multivesicular Body

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Abstract

Although the main action of human hepatitis B immunoglobulin (HBIG) of neutralizing the hepatitis B virus surface antigen (HBsAg) in the serum is known, HBIG is known to be localized in the cell. However, the effect of intracellularly located HBIG is not well elucidated due to the low purity of conventional plasma-derived HBIG (cHBIG). We attempted to clarify the mechanism of action of internalized HBIG using recombinant HBIG (lenervimab).

We used HBsAg cell lines, non-HBsAg cell lines and human HBsAg-producing hepatocytes. Autophagosome lysis pathway related proteins and Rab5, calnexin, giantin, and Rab7 were used to localize HBsAg and anti-HBs-IgG in the cytoplasm with Western blot and confocal microscopy.

Intracellular anti-HBs-IgG (lenervimab and cHBIG) transported by Fc receptor-mediated endocytosis increased the autophagosomes, but there was no change in autolysis. HBsAg and anti-HBs-IgG precipitated in the cytoplasm and co-localized in the multivesicular body. HBsAg secretion in the culture medium was decreased after lenervimab. Simultaneously, the amount of cellular HBsAg increased in the cell lines but decreased in the human hepatocytes. Furthermore, intracellular lenervimab was not easily washed out only in the HBsAg cell lines.

Lenervimab decreases the secretion of HBsAg, and HBsAg-antibody precipitation in the multivesicular body might play an important role.

Introduction

Viral hepatitis is a crucial global health issue. Human hepatitis B virus (HBV) infection is the most common infectious liver disease, with an estimated 257–290 million or 3.5% of the population suffering from chronic hepatitis B virus ^{1,2}. According to the 2016 WHO report, more than 0.7 million people die every year due to the complications associated with HBV infection. The vaccination for HBV in the perinatal period is known to provide effective prophylaxis and is essential for the eradication of HBV. However, the rate of vaccination is still very low in central Africa, the eastern Mediterranean region, and southeast Asia. The incidence of HBsAg positivity is still high in central and southeast Asia (5.3%) and middle Africa (5.6%) ¹. Considering the high rate of HBV-related chronic liver disease, HBV remains a clinical burden.

Hepatitis B immunoglobulin (HBIG) monotherapy was considered an effective prophylactic protocol after liver transplantation in earlier times; however, it might lead to be inefficacious in the G145R mutant and is also expensive ³⁻⁵. Furthermore, additional administration and monitoring of the titer are necessary to maintain adequate serum levels ³. With the development of antiviral nucleos(t)ide analog (NA), the combination of HBIG and NA has the greatest potential and shows the most outstanding results in real-world practice and in the reported data compared to NA monotherapy ^{6,7}. NA monotherapy also showed excellent long-term survival of 85% at 9 years and an undetectable HBV DNA rate of 100% at 8 years ⁸.

However, NA has been known for 9-14% of HBsAg loss of efficacy^{8,9} and nephrotoxicity, especially in patients with ongoing acute kidney injury before liver transplantation (LT)¹⁰

A potent and relatively low-cost HBIG could be a good alternative to overcome the drawbacks of the recent regimen. A recombinant monoclonal HBIG (Lenervimab) developed by GC pharma (Yongin, South Korea) has been reported to have a consistent avidity of variety to cloned S antigens, including the immune escape mutant G145R. Moreover, it does not interfere with antibody binding in HBV with mutations in the S gene sequence that causes resistance to NA⁵. Furthermore, neutralization of the circulating HBV particles and inhibition of re-entry of the virus by binding to HBsAg has been reported^{11,12} however, the action of its intracellular component is not completely understood.

Therefore, we investigated the action of intracellularly located lenervimab in HBV infected cells.

Results

1. Intracellular location of Lenervimab and cHBIG in Hur7 and PLC/PRF/5 via endocytosis

Anti-HBs-IgG (lenervimab and cHBIG) was added to Hur7 cells for 24 h, and anti-HBs-IgG (lenervimab and cHBIG) and control human IgG were treated in PLC/PRF/5 for 24 h. The IF results are shown in Figure 1. Lenervimab and Rab5 (early endosome marker) were co-localized in the cytoplasm, and cHBIG also showed a co-localized pattern in the Hur7 cell line (Figure 1A). However, only the Fab portion, except the Fc portion, and Rab5 were not co-localized and showed a diffuse distribution pattern. In PLC/PRF/5, anti-HBs-IgG (lenervimab and cHBIG) and Rab5 (early endosome marker) were co-localized in the cytoplasm. Control human IgG was located in the cytoplasm with Rab5; however, anti-HBs-IgG was more located in the cytoplasm than control-IgG, and appeared as prominent dot-like patterns. (Figure 1B)

2. Formation of autophagosomes after internalization of anti-HBs-IgG (lenervimab and cHBIG) in HepG2.2.15

Figure 2A shows the prominent dot-like patterns of LC3 and anti-HBs-IgG (lenervimab and cHBIG). However, the intensity graph showed that LC3 and anti-HBs-IgG were not co-localized. (Figure 2B) The Western blot analysis showed the increased intracellular location of the anti-HBs-IgG and HBsAg in the anti-HBs-IgG group. The ratio of LC3 I to LC3 II was significantly increased in lenervimab, similar to that with ammonium chloride treatment (positive control for autophagosome) compared to the cHBIG and control groups. However, p62 (autolysis marker) was not decreased in the anti-HBs-IgG (lenervimab and cHBIG) groups compared to rapamycin (a positive control for autolysis) (Figure 2C).

3. Co-localized precipitation of HBsAg and anti-HBs-IgG (lenervimab and cHBIG) in PLC/PRF/5 and HBsAg

producing human hepatocyte

IF was performed for anti-HBsAg-IgG (lenervimab and cHBIG) and HBsAg markers in PLC/PRF/5 and human hepatocytes producing HBsAg. (Figure 3) Contrary to treatment with control IgG, co-localization of HBsAg and anti-HBs-IgG (lenervimab and cHBIG) was observed in the anti-HBs-IgG treatment group. Moreover, the prominent dot-like staining of HBsAg and IgG was observed only in the anti-HBs-IgG group compared to the control IgG and Fab groups. Dot-like staining was more prominent with lenervimab than with cHBIG. In human hepatocytes (after lenervimab treatment), co-localized HBsAg and lenervimab and dot-like patterns were prominent, similar to those in the PLC/PRF/5 cell line. (Figure 3)

4. Intracellular accumulation of HBsAg and reduced secretion of HBsAg in PLC/PRF/5 by lenervimab

Western blot analysis of intracellular HBsAg and IgG and of the culture supernatant was performed according to the duration (48, 72, and 96 h after lenervimab treatment). The bands of intracellular HBsAg (especially small HBsAg) and lenervimab were prominent 96 h after treatment than that of the control IgG. (Figure 4A) All HBsAg band areas were apparently faint features in the culture supernatant than that of the control IgG. The lenervimab band was less prominent than that of the control IgG in the supernatant. It seems that the continuous treatment of lenervimab in the supernatant effect. (Figure 4B). During the preparation of intracellular proteins, the cells were washed several times. In contrast to the control IgG, HBsAg and IgG remained within the cells in the lenervimab-treated group in PLC/PRF/5.

5. HBsAg and lenervimab in multivesicular body with precipitation in PLC/PFR/5

Figure 5 shows the IF image and intensity graph of HBsAg and endoplasmic reticulum (calnexin), Golgi apparatus (giantin), and multivesicular body (Rab7) markers after treatment with anti-HBs-IgG in PLC/PRF/5. Some amounts of HBsAg and calnexin were co-localized in the control IgG and anti-HBs-IgG groups. However, the dot-like patterns of HBsAg were observed only in the anti-HBs-IgG group than in the control IgG group, and dot-like patterns of HBsAg and calnexin were not co-localized (Figure 5A, 5B). Similarly, some HBsAg and giantin were co-localized in the control IgG and anti-HBs-IgG groups, and dot-like patterns of HBsAg and giantin were not co-localized (Figure 5C, 5D). Rab7, HBsAg, and anti-HBs-IgG were co-localized, and lenervimab cells showed prominent dot-like patterns of HBsAg and lenervimab staining, and the intensity graph showed co-localization of lenervimab and HBsAg (Figure 5E,5F).

Figure 6 shows the schematic life cycle of HBV and the mechanism of blocking the HBV release. As shown in Figure 5, the main location of the antigen-antibody reactions was multivesicular body (MVB), where the main HBsAg assembled organelles after the packaging process. Eventually, the filament type (containing medium and large HBsAg) virus-like particles and HBV could not be released by lenervimab.

Conclusion

The release of HBV is suppressed by lenervimab via an antigen-antibody reaction in the multivesicular body.

Discussion

While NA showed tremendous survival and HBV DNA clearance, anti-HBs-IgG still had an additional effect in controlling the HBV. One of the two main categories of prophylaxis after LT, the combination therapy (HBIG and NA) showed better results than NA alone. NA treatment has several advantages in controlling HBV with different mechanisms of suppression of DNA synthesis and reduced risk of HBIG-related HBsAg mutation, relatively high compliance, and low cost compared to HBIG. However, drug-related toxicity, especially to the kidneys, and a 22.1% chance of immune-escape mutation of HBsAg in patients with NA exposure still exists¹³. HBsAg plays a key role in maintaining the HBV immune tolerance and suppressing the HBV DNA by NA^{14,15}. HBsAg seroclearance is an important requirement for the discontinuation of NA by inducing spontaneous immune control¹⁶. This might be related to the poorer outcomes of the HBIG-free regimen in some patients undergoing transplantation and patients with CHB^{17,18}. However, human plasma fractionated HBIG has drawbacks such as low specific activity, which might lead to loss of efficacy against the G145R mutant and has a high cost³⁻⁵. Lenervimab is a recombinant IgG1-type and anti-HBs-IgG derived from the immunoglobulin genes of the vaccinated persons transferred to Chinese hamster ovary cells, and it has been reported to have more potent activities in drug-resistant variants and all virus genotypes, particularly in the antigenic 'a' determinants⁵. The mechanism of action of lenervimab has been reported to be neutralization of the circulating HBV particles and inhibition of re-entry of the virus by binding^{11,12}. We investigated the mechanism of action of lenervimab in HBV-positive cell lines, focusing on the intracellular portion and differences between lenervimab and cHBIG.

The mechanism of entry of lenervimab and cHBIG is endocytosis, similar to that of the usual immunoglobulin. Figure 1 shows that most of the intracellular location of anti-HBs-IgG was co-localized with the early endosome marker Rab5 in both PLC/PRF/5 and Huh7, as in a previous study¹⁹. However, this finding was not observed in the Fab treated group. Interestingly, the dot-like pattern of anti-HBs-IgG was more prominent in PLC/PRF/5 cells than in Huh7 cells, suggesting that antigen-antibody reactions are related to these findings.

We attempted to elucidate the process after the internalization of anti-HBs-IgG, focusing on the process of autophagy. We hypothesized that the HBsAg-specific antibody (anti-HBs-Ig) after endocytosis could form an HBsAg-antibody complex in the form of autophagosomes and proceed to autolysis. The formation of autophagosomes was more prominent in the lenervimab group than in the control IgG or cHBIG groups; however, sequential autolysis was not obvious. (Figure 2C). Rapamycin, a known autolysis-inducing agent, was added after lenervimab treatment; however, autolysis after autophagosome formation did not occur (data not shown). Furthermore, LC3 (an autophagosome

marker) did not co-localize with anti-HBs-IgG (Figure 2B). In general, autophagosome formation is induced by ER stress through phagopore-initiating processes.^{20,21} In this study, an antigen-antibody reaction did not occur inside the autophagosome; however, the formation of autophagosomes was increased. The possible mechanism of increased autophagosomes is ER stress induced by lenervimab in an HBsAg-producing cell line. Therefore, antigen-antibody reactions increase the ER stress and induce autophagosome formation through the phagopore-initiating process²². The interference in interactions between MVB and autophagosomes might be a possible reason. MVB is related to autophagosomes making amphisomes that can fuse with lysosomes, which are known to generate autophagolysosomes²³. Accumulation and altered function of MVB after antigen-antibody reactions might be related to increased autophagosome formation in the absence of autolysis.

We noticed that the dot-like patterns of co-localized HBsAg and anti-HBs-IgG were observed only in the HBsAg-producing cell lines. When only the Fab portion was treated, the dot-like patterns were not observed. (Figure 3). According to this result and previous studies, the Fc portion is necessary for the stabilization of the antigen-antibody reactions after endocytosis and regulation of lysosomal activity and endosomal sorting complex required for transport (ESCRT).^{19,24} Therefore, the dot-like patterns are related to the Fc portion after antigen-antibody reactions, and the Fc portion seems to play an important role in maintaining a strong binding affinity for HBsAg or adjacent Ig.

In the cHBIG-and lenervimab-treated groups, accumulation of HBsAg in the intracellular portion and reduced HBsAg secretion were observed in the supernatant. Previous studies also showed that elimination of the Fc portion resulted in the secretion of HBsAg in PLC/PRF/5¹⁹. Even after washing the cell culture plate, some HBsAg and lenervimab remained intracellularly. (Figure 4) Inhibition of HBsAg by lenervimab was observed in human hepatocytes, but intracellular HBsAg levels were slightly decreased. A previous study showed similar findings. Further studies are necessary to clarify the differences between HBsAg-producing cell lines and infected human hepatocytes.

Eventually, the locations of these antigen-antibody complexes were co-localized with Rab7 (MVB marker). This result is meaningful in terms of inhibition of the HBV infectivity, considering the pathways of the subviral HBV particles. Small HBsAg with spheres (without infectivity) secretion usually occurs via the ER-Golgi network, is released by the general secretory pathway, does not accumulate in general, and does not significantly inhibit the production and secretion of small HBsAg (spheres) in an inhibited MVB biogenesis cell line²⁵. However, virions containing medium or large HBsAg, which have infectivity, require the ESCRT dependently via MVB for secretion²⁵. HBV secretion is regulated through the activation of endocytic and autophagic compartments mediated by Rab7 stimulation, and MVB has been known to participate in the final stage of HBV maturation and release^{25,26}. Silencing either Rab5 and Rab7 results in the inhibition of HBV infection showed similar result of these findings²⁷. Accumulated lesions of virions in MVB and antigen-antibody complexes occur in MVB, which manifests as Rab7, as shown in this study. Accumulation of HBsAg in MVB is not only unable to secrete the HBV particles themselves, but also has no infectious ability because medium and large HBsAg that bind with heparin sulfate

proteoglycans, which means only secretion of HBV particles could not have infectious ability²⁸. In chronic HBV infection, high rates of virus-like particles consisting of medium or large HBsAg are major obstacles in triggering effective immune responses and subsequent virus clearance²⁹. Based on our findings, we suggest that antigen-antibody reactions in MVB compromise the HBsAg secretory pathway of virion-or filament-type VLPs (Figure 6). Monoclonal antibodies with strong and multisite affinity, such as lenervimab, showed a stronger effect than cHBIG.

We focused on the mechanism of lenervimab and HBsAg, which were confirmed in Rab7 marker-positive organelles. It appears that not only suppression effect the HBV release of anti-HBs-IgG, but also the main location of antigen-antibody reaction is MVB, where the main HBV assembly organelles have not yet been reported.

Methods

Cell lines and cell culture

We used five human hepatoma cell lines from Korean cell line bank (KCLB) and Merck KGaA. Huh7 (KCLB No. 60104), HepG2 (KCLB No. 88065) as HBV negative, PLC/PRF/5 (KCLB No. 28024), HepG2.2.15 (Merck KGaA, No. SCC249), and Hep3B (KCLB No. 88064) expressing HBsAg. The cell lines were cultured in a 37°C, 5% CO₂ incubator and Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS, Thermo Scientific Hyclone) and 1xAntibiotic-Antimycotic (Anti-Anti).

Human Hepatocyte Isolation

Human hepatocytes from patients with high titers of HBsAg and HBV DNA in the blood were used. Approximately 50–70 g of liver tissue from a patient infected with hepatitis B virus and positive for HBsAg was obtained. After connecting two or three catheters filled with William's media to a large vessel exposed to a cross-section of the liver tissue, perfusion solution A [Hanks' balanced salt solution (HBSS) 250 ml + hydroxyethyl piperazine-ethane-sulfonic acid (HEPES) 2.5 ml + antibiotics 2.5 ml + EDTA 250 µl] was perfused through a peristaltic pump at a flow rate of 30 ml per minute per catheter. After approximately 10 min, a perfusion solution B (HBSS 250 ml + HEPES 2.5 ml + antibiotics 2.5 ml +collagenase 0.125 g) was used for perfusion for another 10 min. After removing the catheter from the liver tissue that had turned yellow due to decomposition, the liver tissue was transferred to another bowl, and cold William's medium was poured. A knife was used to obtain the enzymatically degraded tissue cells.

Immunoglobulins

Three immunoglobulins [control human IgG (Sigma, Dorset, UK), conventional plasma-derived hepatitis B immunoglobulin (cHBIG, GC pharma, Yongin, Korea), and recombinant hepatitis B immunoglobulin and

its Fab portion (lenervimab, GC pharma, Yongin, Korea)] were diluted with Dulbecco's Phosphate Buffered saline (DPBS) or media corresponding to the cell line and used in various concentrations from 0.25 mg/ml to 10 mg/ml. For immunofluorescence and western blotting, the cell lines were treated from 15 min to 96 h and 48 h, respectively, in a 37°C, 5% CO₂ incubator.

Immunofluorescence

Each cell line was cultured in an 8-well chamber slide (Nunc™ Lab-Tek™) at a density of 0.3x10⁵ cells/well, or a cover glass was placed in a 24-well cell culture plate and seeded at 0.5 × 10⁵ cells/well. After 24 h, control human IgG, cHBIG, or lenervimab were treated in a 5% CO₂ incubator at 37°C for 15 min to 48 h, and then the cells were fixed with 3.7% paraformaldehyde for 15 min, followed by incubation for 15 min using 0.2% Triton X-100. Blocking was performed with 1% bovine serum albumin (BSA) for an hour. The primary antibodies used were mouse anti-HBsAg and mouse anti-Rab5 (early endosome marker), rabbit anti-LC3 (autophagy marker), mouse anti-calnexin (ER marker), mouse anti-giantin (Golgi marker), and Rab7 (multivesicular body marker) in a ratio of 1:100. For the corresponding secondary antibody, an antibody conjugated with fluorescence, goat anti-mouse IgG (488), goat anti-human IgG (568), and horse anti-rabbit IgG (568) were used in a ratio of 1:200. In addition, wheat germ agglutinin (633) and Alexa Fluor-phalloidin 488 were used in a ratio of 1:500. This immunofluorescence (IF) sample was observed using a Leica TCS SP8 confocal microscope, and the corresponding fluorescence was used.

Western Blot

Each cell line was seeded on a 6 well cell culture plate, treated with necessary IgG or reagents, and then the cells of each well were scraped and lysed on ice for 30 min after treatment with radioimmunoprecipitation assay lysis buffer and proteinase inhibitor. The lysed cells were centrifuged at 4°C for 30 min to obtain the supernatant. A 5x sample buffer was added, boiled for 5 min, and cooled on ice.

Western blotting was performed for 55 min at 130 V and 300 mA using 4–20% precast gel and then transferred to the polyvinylidene fluoride membrane at 80 V and 300 mA for 2 h at 4°C in a cold room. This was blocked with 5% BSA and washed with tris buffered saline. The primary antibody was incubated in a cold room at 4°C overnight, and the secondary antibody was incubated at room temperature for 1 h. The results were measured and analyzed using an LAS 4000 instrument.

As described in figure, each protein was loaded on a gel and transferred to one sheet of membrane. The proteins were detected using each antibody after cutting the membrane suitable size of the interested protein. In case of HBsAg, we used entire membrane to detect the various size of proteins consisting of HBsAg such as small, medium and large (Figure 4) (Supplementary file)

Cell culture medium protein assay

1) Western blot after polyethylene glycol (PEG) down

In order to characterize the proteins in the cell culture medium by Western blotting, the culture medium was collected separately and then centrifuged at 8000 rpm. The supernatant (2 ml of supernatant was mixed with 1 ml of 40% PEG, 400 µl of 4M NaCl, and DMEM to adjust to a total volume of 4 ml. This was mixed with a rotator at 4°C overnight, and the pellet was centrifuged at 4850 rpm at 4°C and used for Western blotting.

2) Enzyme-linked immunosorbent assay

The PLC/PRF/5 cell line was seeded into a 6 well plate. After 24 h, FBS free DMEM was added in each plate and, CHBIG or lenervimab, and Fab portion of lenervimab were added. and After 48 h, the supernatant was replaced with FBS-free DMEM. The supernatant was obtained every 12, 24, and 48 h, centrifuged at 10,000 rpm for 20 min at 4°C, and the supernatant was used for enzyme-linked immunosorbent assay (ELISA).

A human HBsAg ELISA kit was used. A blank sample was added to each well and incubated at 37°C for 90 min. After removing the liquids in the well and treating the supernatant with 100 µl of biotinylated detection antibody, it was gently tapped to mix well and incubated again at 37°C for 1 h. After washing each well three times with wash buffer, 100 µl of Horseradish peroxidase conjugate working solution was added and incubated at 37°C for 30 min. After washing each well five times with wash buffer, 90 µl of the substrate was treated and incubated at 37°C for 15 min. After 50 µl of the stop solution was added, the optical density was measured at 450 nm using a microplate reader.

Ethics

This study followed the ethical guidelines of the Declaration of Helsinki and was approved by the institutional review board of Seoul National University Hospital (institutional review board no 1401-081-550, 1701-004-819). In this study, we used human hepatocyte form patient sample after resection and we received informed consent with IRB approval (IRB number was described above). The dataset from qualified researchers trained in human subject confidentiality protocols may be sent to the Seoul National University Hospital institutional review board at cris@bri.snuh.org (<https://cris.snuh.org>).

Declarations

Acknowledgement

Kyung Chul Yoon and Sooin Seo contributed equally as co-first authors

Author contributions

KC Yoon; drafting of the manuscript, analysis and interpretation, acquisition of data

S Seo; drafting of the manuscript, technical and material support, analysis and interpretation, acquisition of data

K-W Lee; study concept and design, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, obtained funding, study supervision

SC Oh; technical and material support

MY Park; technical and material support

SK Hong; data analysis, tissue sampling

YR Choi; analysis and interpretation, helped supervise the project

N-J Yi; analysis and interpretation, helped supervise the project

K-S Suh; analysis and interpretation, helped supervise the project

Disclosure

Kyung Chul Yoon; don't have any conflict of interest

Soojin Seo; don't have any conflict of interest

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Figures

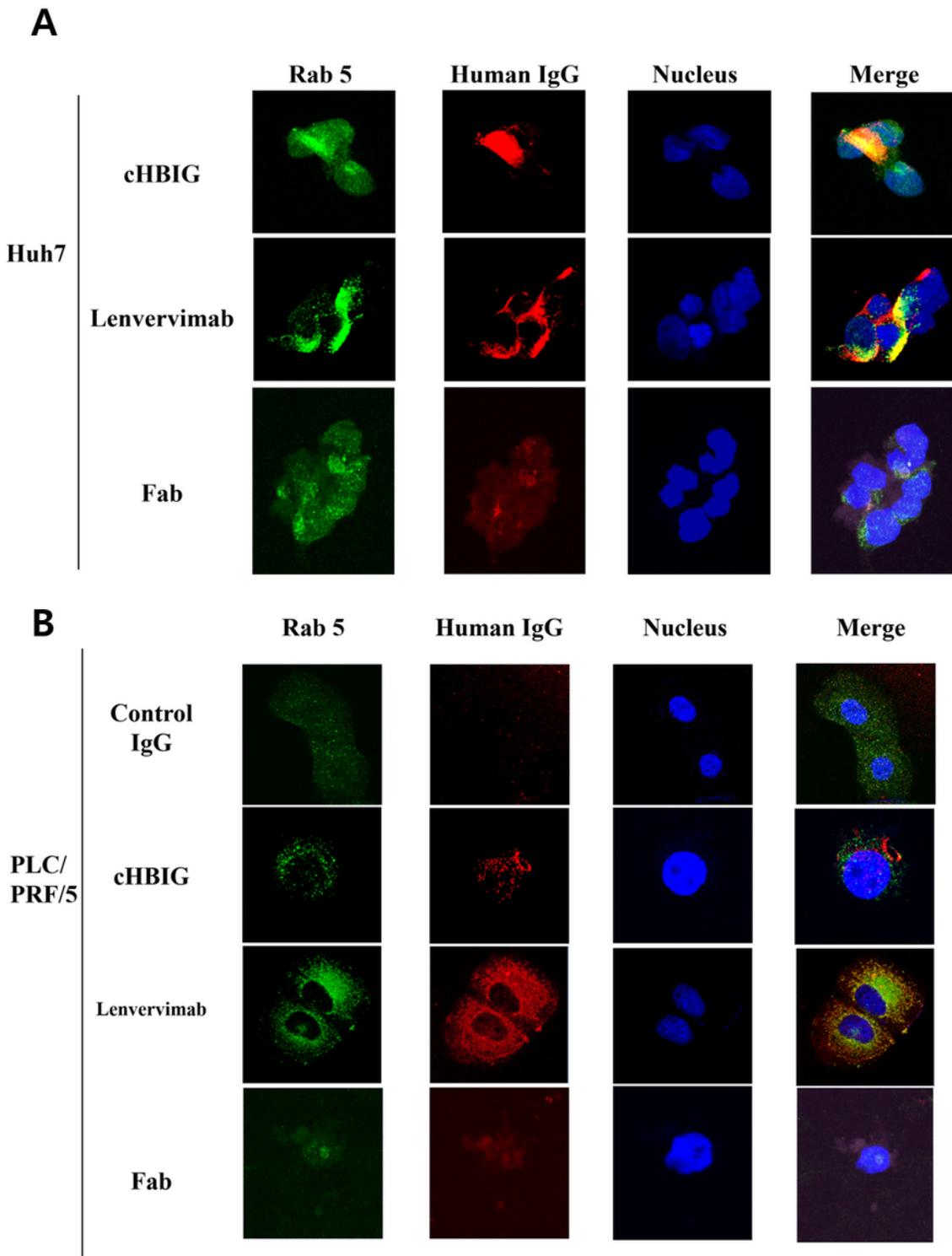


Figure 1

Immunofluorescence images of immunoglobulin marker and Rab5 (early endosome marker) in Huh7 and PLC/PRF/5 cell line after treatment of anti-HBs-Ig (lenervimab and cHBIG) for 24 hours. (A) Rab5 and anti-HBs-IgG were co-localized (showed with yellow color), however Fab portion (except Fc portion) and Rab5 were not co-localized with diffuse distributed pattern in Huh7. (B) Co-localization of Rab5 and immunoglobulin (control IgG anti-HBs-IgG) in PLC/PRF/5 were showed, however Fab portion (except Fc

portion) and Rab5 were not co localized. Anti-HBs-IgG is located in cytoplasm more than control IgG and the dot like patterns were more prominent in anti-HBs-IgG group.

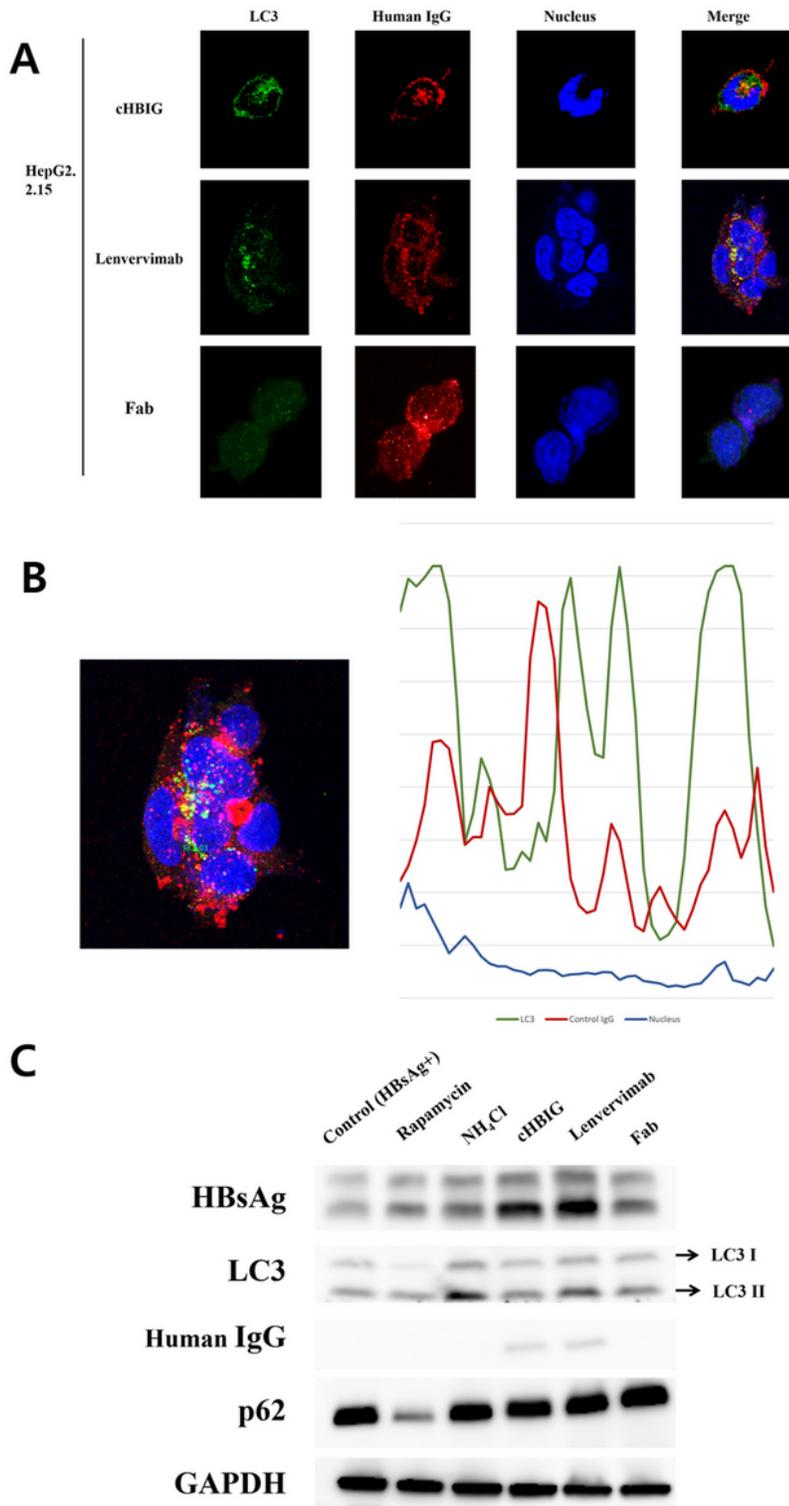


Figure 2

Autophagosome formation but autolysis was not observed. (Each protein was loaded on a gel and transferred to one sheet of membrane. The proteins were detected using each antibody after cutting the membrane suitable size of the interested protein. supplementary file serves entire membranes) (A) LC3

and anti-HBs-Ig (lenervimab and cHBIG) were prominent with dot like patterns. (B) However, the intensity graph showed LC3 and anti-HBs-Ig were not co-localized. (C) Western blot analysis of the human Ig and HBsAg, LC3, P62. Intracellular location of anti-HBs-IgG and HBsAg and the increased ratio of LC3 I to LC3 II was prominent in lenervimab similar to ammonium chloride (positive control for autophagosome). However, the P62 was not decreased in anti-HBs-Ig compared to the rapamycin (positive control for autolysis).

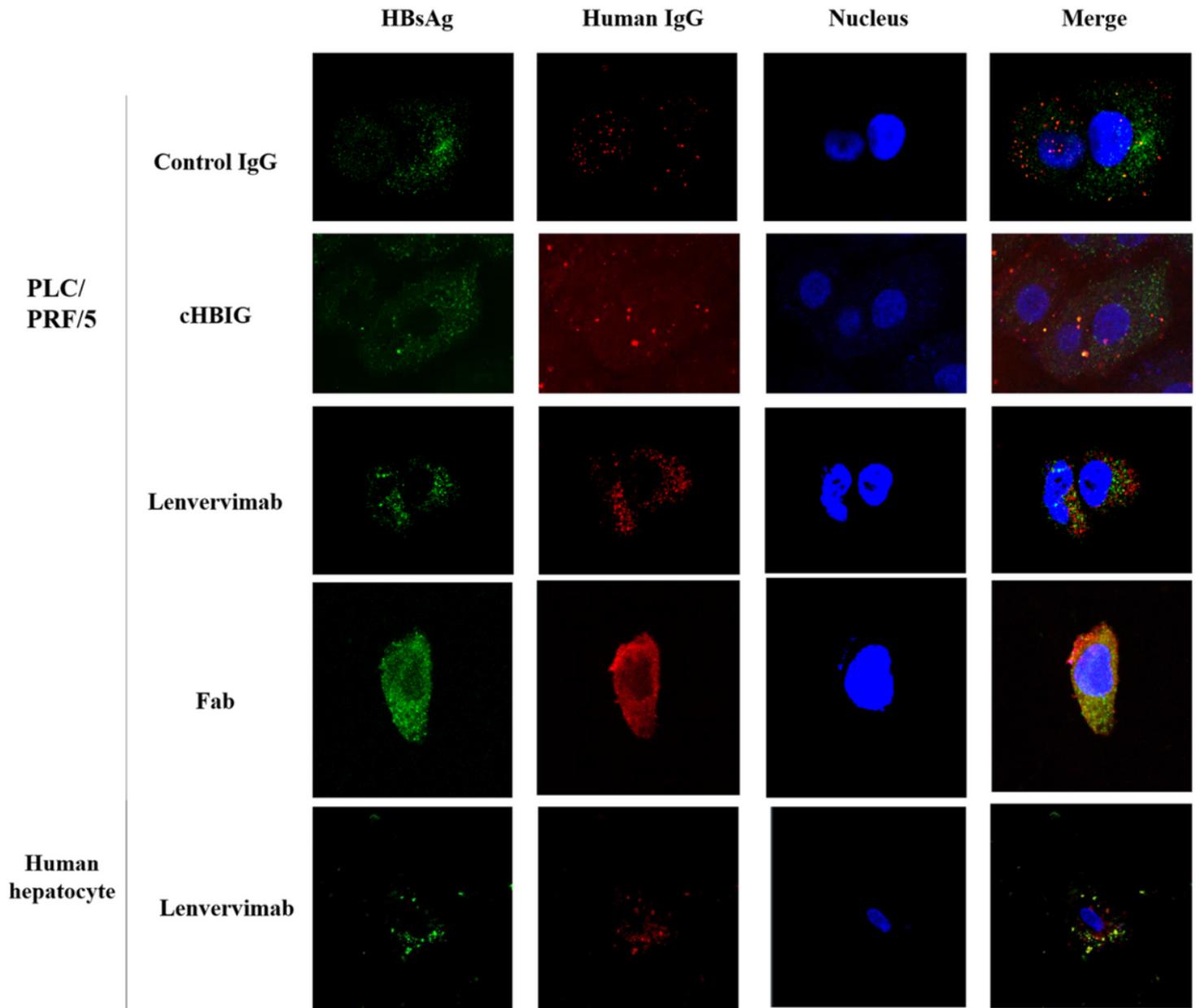


Figure 3

Immunofluorescence images of immunoglobulin marker and HBsAg in PLC/PRF/5 and human hepatocyte isolated from the patient with HBsAg and DNA in the blood. Co-localization of HBsAg and anti-HBs-IgG (lenervimab and cHBIG) was observed and dot like pattern is prominent in anti-HBs-IgG group. Lenervimab formed more dot like patterns than cHBIG. Fab portion except Fc portion

immunoglobulin, dot like pattern was not prominent without co-localization with HBsAg. Co-localization of HBsAg and lenervimab and dot like pattern were also observed in HBsAg producing human hepatocyte and dot like pattern was prominent similar as anti-HBs-Ab treating group in PLC/PRF/5.

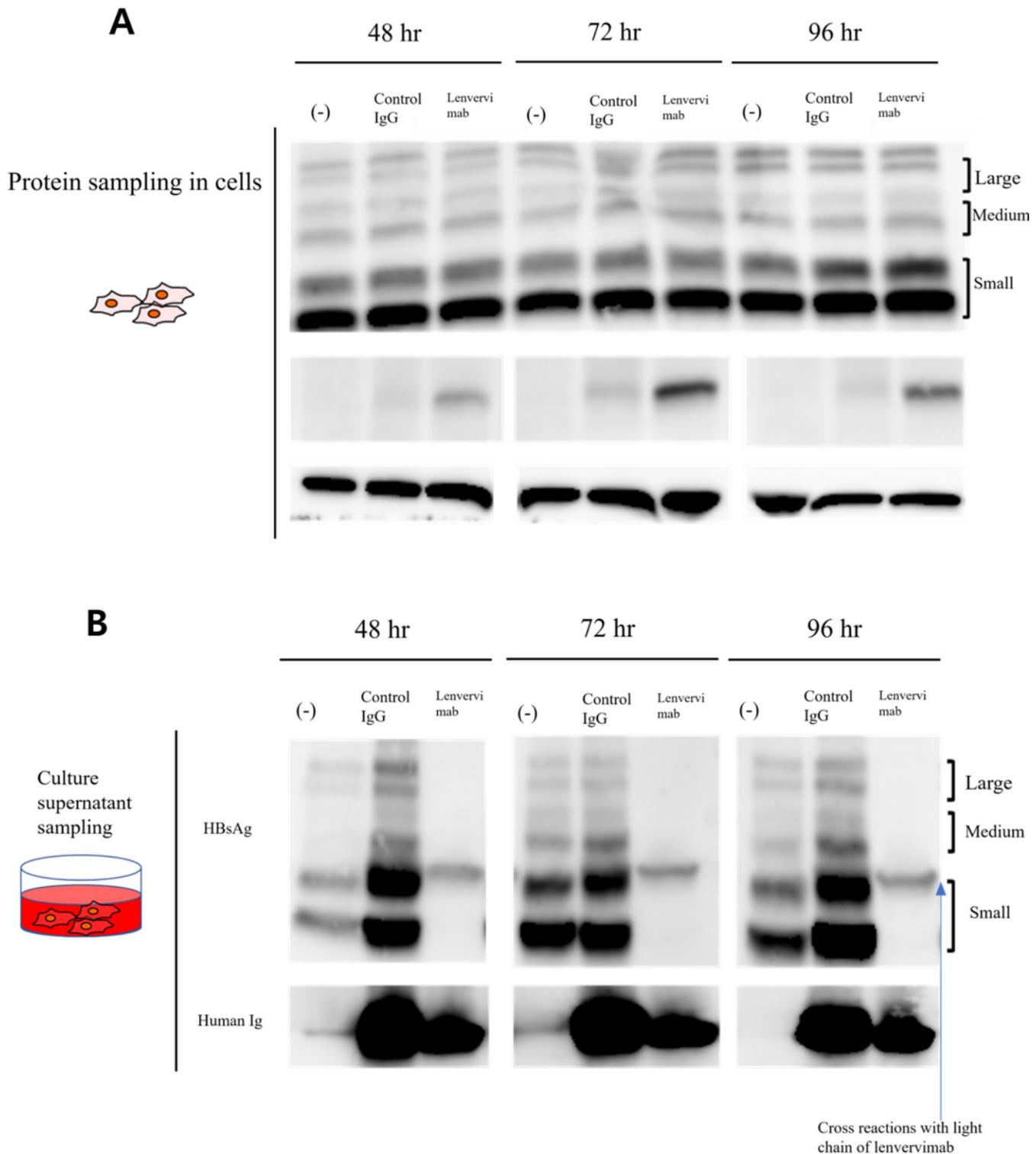


Figure 4

Western blot analysis of HBsAg and immunoglobulin in PLC/PRF/5 cell lines after control human Ig and lenervimab. Control immunoglobulin and lenervimab was treated for 96 hours in PLC/PRF/5. (Each

protein was loaded on a gel and transferred to one sheet of membrane. The proteins were detected using each antibody after cutting the membrane suitable size of the interested protein. In case of HBsAg, we used entire membrane to detect the various size of proteins consisting of HBsAg such as small, medium and large supplementary file serves entire membranes) (A) Intracellular accumulation HBsAg and lenervimab was prominent in lenervimab treated cells comparing to control Ig (B) Reduced HBsAg in supernatant after lenervimab treatment and band of Ig in lenervimab was less prominent compared to control Ig.

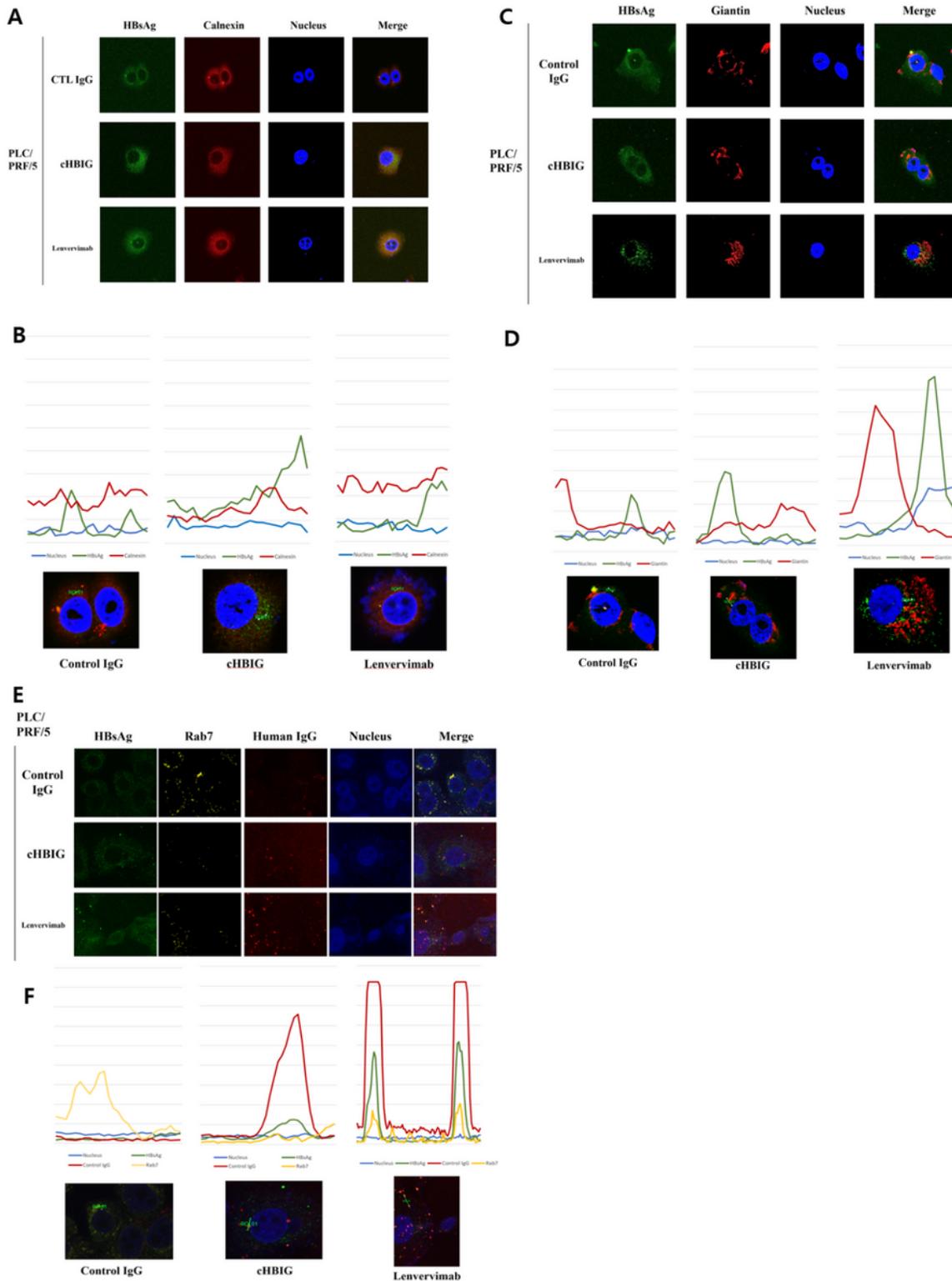


Figure 5

Immunofluorescence image of HBsAg and endoplasmic reticulum(calnexin), golgi apparatus (giantin), multivesicular body (Rab7) markers after treating anti-HBs-Ig in PLC/PRF/5 (A)(B) Some part of HBsAg and calnexin were co-localized in control Ig and anti-HBs-Ig group. The dot like patterns of HBsAg was seen only in anti-HBs-IgG group and the dot like patterns of HBsAg and calnexin were not co-localized (C) (D) Some part of HBsAg and giantin were co-localized in control Ig and anti-HBs-IgG group and the dot like patterns of HBsAg and giantin were not co-localized (E)(F) Rab7 and HBsAg, anti-HBs-IgG were co-localized and treating lenervimab cells showed more prominent dot like patterns of HBsAg and lenervimab and the intensity graph also showed co-localized of lenervimab and HBsAg, Rab7

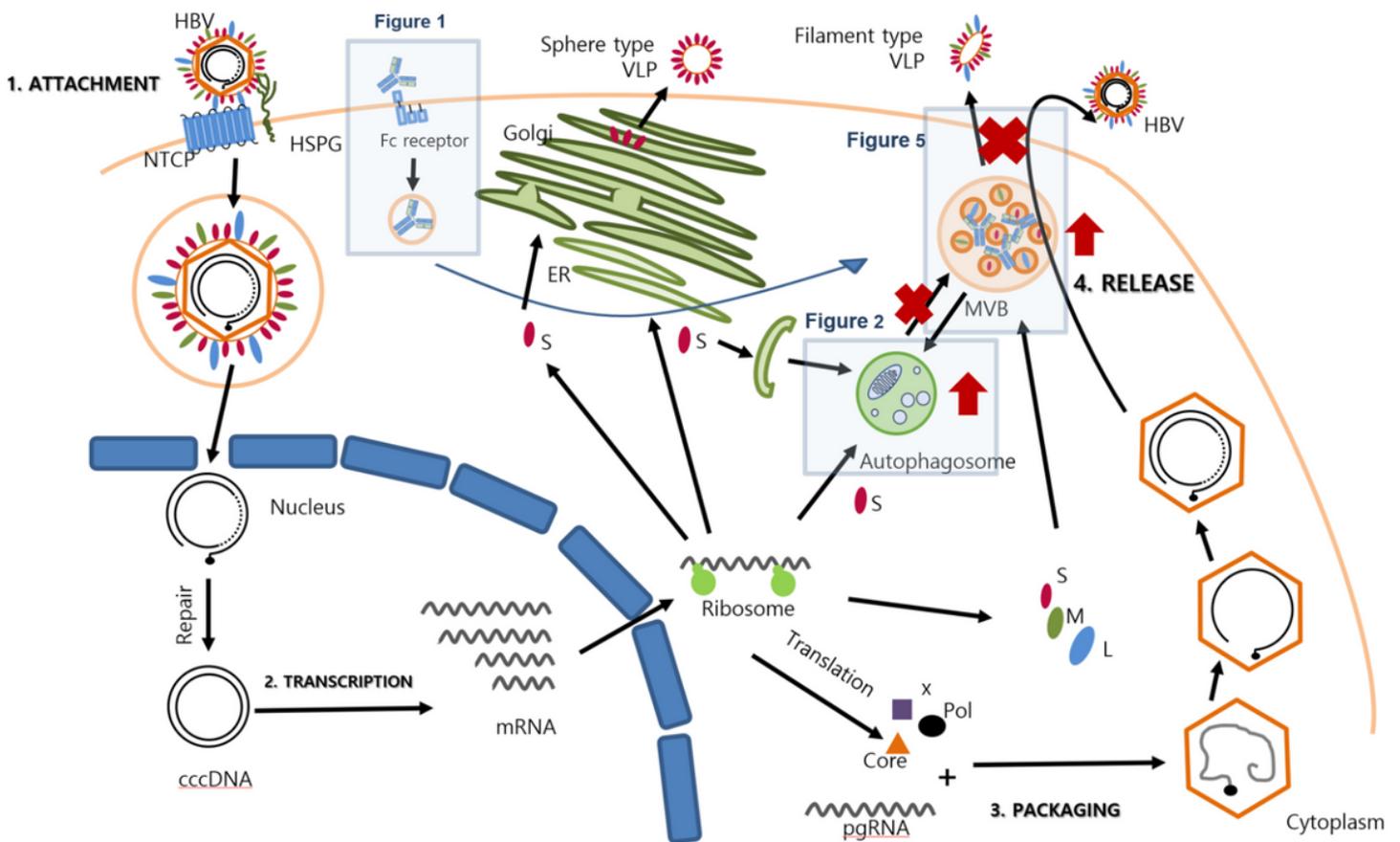


Figure 6

Schemes of life cycle of HBV and related figures of this study. the main location of the antigen-antibody reactions was multivesicular body (MVB), where the main HBsAg assembled organelles after the packaging process. Eventually, the filament type (containing medium and large HBsAg) virus-like particles and HBV could not be released by lenervimab cccDNA; covalently closed circular DNA, NTCP; sodium/taurocholate co-transporting polypeptide, HSPG; heparan sulfate proteoglycan, MVB; multivesicular body, pgRNA; pregenome RNA, VLP; virus like particle

Supplementary Files

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