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# *Helicobacter pylori* cytotoxin-associated gene A impairs the filtration barrier function of podocytes *via* p38 MAPK signaling pathway

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***Helicobacter pylori*** **(Hp) specific antigens were found deposited in the glomeruli in some kidney diseases. However, the underlying molecular mechanisms remain to be elucidated. The aim of this study was to investigate the effect of cytotoxin associated gene A protein (CagA), a key virulence factor of Hp, on mouse podocytes. Cells were cultured and treated with recombinant CagA protein. The expression of the tight junction protein ZO-1 and p38 MAPK signaling pathway activation were measured with real-time RT-PCR and western blotting. The filtration barrier function of podocytes was evaluated with albumin influx assay. CagA decreased the expression and membrane distribution of ZO-1, impaired the filtration barrier function of podocytes, while activating p38 MAPK signaling pathway in these cells. Selective p38 MAPK inhibition partly prevented CagA-induced filtration barrier dysfunction of podocytes through ameliorating ZO-1 downregulation. Taken together, the results suggested that CagA, at least *via* p38 MAPK signaling pathway, may induce podocyte injury. Anti-Hp therapy may be beneficial for the treatment of kidney diseases related to Hp antigen deposition.**

**Key words**: CagA, ZO-1, p38 MAPK, podocyte, proteinuria

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**Abbreviations**: Hp, *Helicobacter pylori*; CagA, cytotoxin associated gene A protein; VacA, vacuolating toxin A; MN, membranous nephropathy; HSPN, Henoch Schonlein Purpura nephritis; LN, lupus nephritis; ECL, enhanced chemiluminescence; MALT, mucosa-associated lymphoid tissue

## INTRODUCTION

*Helicobacter pylori* (Hp), a gram-negative bacterium, was reported to infect more than 50% of the population in Asia (Eusebi *et al*., 2014). A number of virulence factors of Hp were identified such as cytotoxin-associated gene A (CagA), vacuolating toxin A (VacA) (Testerman & Morris, 2014). Among them, CagA is the most important and best-studied virulence factor, responsible for alterations in multiple intracellular signaling pathways and consequently exerting a great influence on host cell function (Censini *et al*, 1996; Covacci *et al*, 1993; Testerman & Morris, 2014).

Hp infection is not only associated with some gastrointestinal diseases such as peptic ulcer disease, gastric cancer, but also plays a role in the pathogenesis of several extragastric diseases such as cardiovascular diseases, diabetes mellitus and autoimmune diseases (Franceschi *et al*., 2014). Recently, some evidence suggested a potential connection between Hp infection and particular kidney diseases. In patients with membranous nephropathy (MN), Henoch Schonlein Purpura nephritis (HSPN) or lupus nephritis (LN), Hp-specific antigens were found deposited along the glomerular capillary walls (Nagashima *et al*., 1997; Yang *et al*., 2009; Li *et al*., 2013). While Hp eradication treatment induced significant remission of proteinuria in patients with primary glomerulonephritis including MN, the underlying mechanisms of the Hp-specific antigens deposition remain unknown (Dede *et al*., 2015).

Podocyte injury plays a crucial role in the development of proteinuria (Brinkkoetter *et al*., 2013). Tight junction protein ZO-1, an important component of podocyte slit diaphragm, is indispensable for maintaining the integrity of glomerular filtration barrier (Itoh *et al*., 2014). The suppression of ZO-1 impairs the formation of podocyte slit diaphragm and consequently triggers the onset of proteinuria (Itoh *et al*., 2014). Whether the Hp-specific antigens deposited in glomeruli may induce abnormal expression and/or distribution of ZO-1 and subsequent podocyte injury remains to be elucidated. Therefore, the present study was designed to investigate the effect of CagA, the key virulence factor of Hp, on podocyte injury and proteinuria and its possible mechanisms.

## MATERIALS AND METHODS

**Cell culture and experimental protocol**. The conditionally immortalized mouse podocyte cell line was kindly provided by Dr Peter Mundel (Mount Sinai School of Medicine, New York, NY, USA) and cultured as described previously (Jing *et al*., 2015). Recombinant CagA protein which originates from Hp strain ATCC 43504 and contains three EPIYA motifs, was purchased from Shanghai Linc- Bio Science Co. Ltd (Shanghai, China). For dose-dependent experiments, differentiated podocytes were incubated with different concentration (0, 5, 10 and 20 μg/ml) of recombinant CagA protein for 72 h; for time-dependent experiments, cells were incubated with the optimal concentration of recombinant CagA protein for 12, 24, 48 and 72 h. Then the optimal concentration and incubation time were used in the subsequent experiments. To investigate the role of p38 MAPK signaling pathway in podocyte injury, differentiated podocytes were pretreated with 5 μM SB203580, a p38 inhibitor (ApexBio, TX, USA) for 1 h prior to being incubated with recombinant CagA protein.

**CCK-8 assay.** Cell viability was evaluated with CCK-8 assay (Signalway Antibody, MA, USA). 3000 cells per

well were plated in 96-well plate, cultured until attachment, then treated with different concentration (0, 5, 10 and 20 μg/ml) of recombinant CagA protein. At 0 h or 72 h of the treatment, 10 μl of CCK-8 solution was added to each well of the 96-well plate and the plate was incubated for 1 h at 37°C. Then the absorbance was measured at 450 nm using a spectrophotometer.

**Real time RT-PCR**. Total RNA was extracted from cells using Trizol reagent (Invitrogen, CA, USA). cDNA synthesis from total RNA was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, MA, USA). Resulting cDNA (1 μg) was amplified in real time, in 25 μl reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems, CA, USA), appropriate primer pairs and water using ABI 7300 thermocycler (Applied Biosystems, CA, USA). The reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. The expression of target gene was normalized to the reference gene GAPDH in the same cDNA sample. The following primers were used: ZO-1: forward, 5'-AGGCTACCTTTGTATTCTC-3', reverse, 5'-TAGGGCACAGTATTGTATC-3'; p38 MAPK: forward, 5'-GTGTTCACACCCGCAAGGTC-3', reverse, 5'-CGGTCAGCTTCTGGCACTTC-3'; GAPDH: forward, 5'-ATCACTGCCACCCAGAAG-3', reverse, 5'-TCCACGACGGACACATTG-3'. The results were analyzed using $2^{-\Delta\Delta CT}$ method.

**Western blotting**. Total and membrane proteins were extracted using RIPA buffer (Solarbio, Beijing, China) and the membrane protein extraction kit (Beyotime, Shanghai, China), according to the manufacturer's instructions, respectively. Then the extracted proteins were resolved using SDS-PAGE and transferred to nitrocellulose (NC) membranes (Millipore, MA, USA). The NC membranes were blocked with 5% non-fat milk and incubated with primary antibodies overnight, followed by incubation with appropriate secondary antibodies for 1 hour at room temperature. The images were visualized using an enhanced chemiluminescence (ECL) detection system (Millipore, MA, USA). The primary antibodies used were as follow: anti-ZO-1 (1:50; Abcam, MA, USA), anti-p38 (1:1000; Cell Signaling Technology, USA), anti-p-p38 (1:1000; Cell Signaling Technology, MA, USA), anti-β-actin (1:1500; Cell Signaling Technology, MA, USA), and anti-GAPDH (1:1500; Cell Signaling Technology, MA, USA).

**Albumin influx assay**. To evaluate the filtration barrier function of podocytes, albumin influx assay was performed as described previously (Kumar *et al.*, 2010). Briefly, differentiated podocytes ($5\times10^4$) were seeded onto the collagen-coated transwell filters (0.4-μm pore; Corning, NY, USA) at the top chamber. Then podocytes were serum-starved overnight (for the cell confluency level before CagA treatment see Fig. 1A) and treated with 20 μg/ml of recombinant CagA protein for 72 h. To determine the role of p38 MAPK signaling pathway in the filtration barrier function of podocytes, differentiated podocytes were pretreated with 5 μM SB203580 for 1 h prior to being treated with recombinant CagA protein. After treatment with recombinant CagA protein, podocytes were washed twice with PBS supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ to preserve the cadherin-based junctions. Then the top chamber was refilled with 0.15 ml of RPMI 1640 and the bottom chamber with 1 ml of RPMI 1640 supplemented with 40 mg/ml of bovine serum albumin. The podocytes were incubated at 37°C for 1 h. Then a small aliquot of medium from top chamber was collected and the albumin concentration was mesured using the bicinchoninic acid protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

**Statistical analysis**. All the experiments were repeated at least three times. All data were presented as mean ±S.D. Differences between groups were evaluated by independent Student's *t*-test using SPSS version 16.0 (SPSS Inc., USA). $P<0.05$ was considered significant.

## RESULTS

### Dose- and time-dependent effect of CagA on the protein expression of ZO-1 in mouse podocytes

Within the range of 5–20 μg/ml, CCK-8 assay demonstrated that CagA had no significant influence on cell viability in mouse podocytes (Fig. 1B). However, CagA decreased the protein expression of ZO-1 in mouse podocytes in a dose-dependent manner (Fig. 2A and B). Accordingly, 20 μg/ml was chosen as the optimal concentration of CagA for subsequent studies. When compared to the control group, CagA treatment decreased the protein expression of ZO-1 in a time-dependent

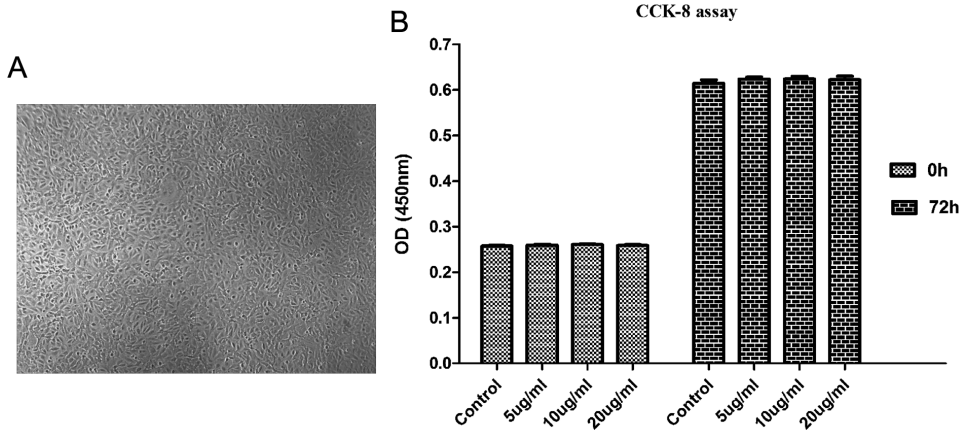


**Figure 1. Cell confluency level and CCK-8 assay results.**
(**A**) Podocytes at 100% confluence before CagA treatment photographed under an inverted microscope. Original magnification, ×100. (**B**) At 0 h or 72 h, CCK-8 assay showed that there was no significant difference in cell viability between podocytes treated with different concentration (0, 5, 10 and 20 μg/ml) of recombinant CagA protein.

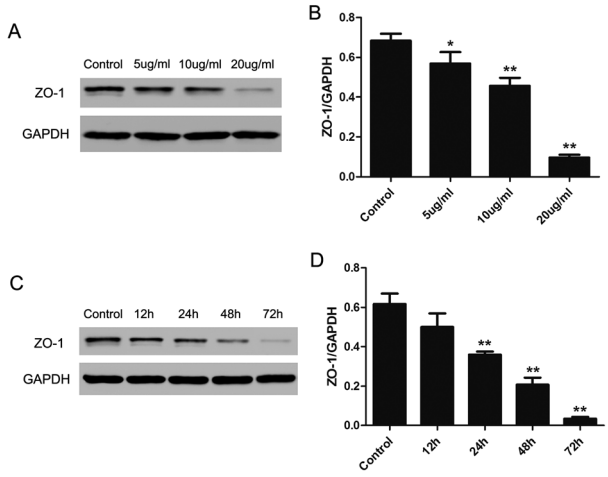


**Figure 2. Dose- and time-dependent decrease of ZO-1 protein expression in CagA-treated mouse podocytes.**
(**A** and **B**) Western blotting (**A**) and quantitative analysis (**B**) showed that when compared to control, CagA significantly decreased ZO-1 protein expression in a dose-dependent manner. (**C** and **D**) Meanwhile, ZO-1 protein expression was also markedly reduced by CagA treatment in a time-dependent manner. *$p$<0.05, compared to control; **$p$<0.01, compared to control.

manner from 24 to 72 h, while there was no statistical difference at 12 h (Fig. 2C and D).

### Influence of CagA on the expression of total and membrane ZO-1 as well as on the filtration barrier function of mouse podocytes

In comparison to the control group, the mRNA expression of ZO-1 was significantly decreased after CagA treatment for 72 h (Fig. 3A). Consistently, with the mRNA change the protein levels of total and membrane ZO-1 were also downregulated (Fig. 3B and C). Moreover, albumin influx assay was used to evaluate the filtration barrier function of mouse podocytes. CagA treatment resulted in an increased albumin influx across the podocyte monolayer at 72 h, which suggested an impaired filtration barrier function of podocytes (Fig. 3D).

### Effect of CagA on p38 MAPK signaling pathway in mouse podocytes

When compared to the control group, the level of p38 MAPK mRNA was significantly increased after CagA treatment for 72 h, while SB203580 pretreatment partly diminished this effect (Fig. 4A). Consistently with the mRNA level change, the protein levels of total and phosphorylated (activated) p38 MAPK were also increased after CagA treatment for 72 h, while SB203580 pretreatment partly prevented these changes (Fig. 4B, C and D).

### Impact of p38 MAPK inhibition on the expression of ZO-1 and filtration barrier function of mouse podocytes incubated with CagA

Selective p38 MAPK inhibition *via* SB203580 pretreatment significantly increased the mRNA level of ZO-1 which was suppressed by CagA treatment, although the level was still statistically different from the control group (Fig. 5A). Similar results were observed for the ZO-1 protein expression (Fig. 5B and C). Moreover, albumin influx assay showed that SB203580 pretreatment notably attenuated albumin influx across the podocyte

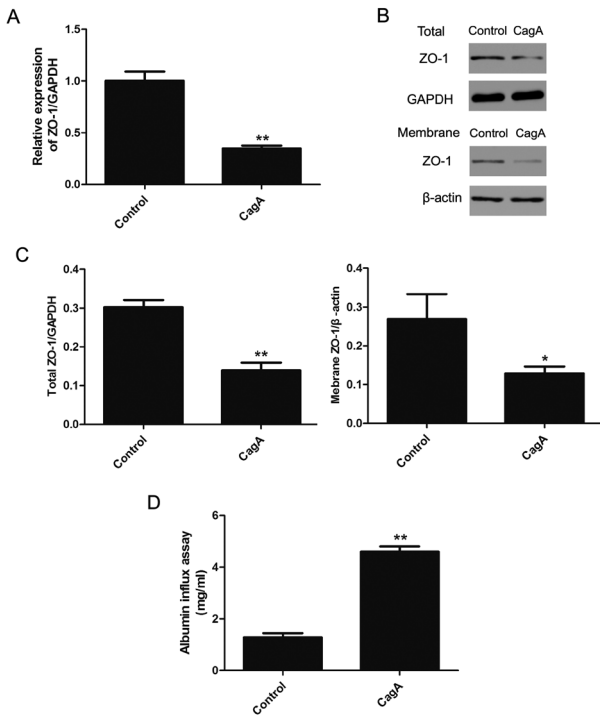


**Figure 3. CagA decreased the expression and membrane distribution of ZO-1, impaired filtration barrier function of mouse podocytes at 72 h of incubation.**
(**A**) Real time RT-PCR showed that when compared to control group, CagA significantly downregulated the mRNA expression of ZO-1. (**B** and **C**) Western blotting (B) and quantitative analysis (**C**) indicated that both the total and membrane ZO-1 protein expression was notably decreased by CagA treatment. (**D**) Albumin influx assay showed that CagA markedly increased albumin influx across the podocyte monolayer, which suggested podocyte filtration barrier injury. *$p$<0.05, compared to control; **$p$<0.01, compared to control.

monolayer which was induced by CagA treatment, although it was still statistically different from the control group (Fig. 5D).

## DISCUSSION

Among the virulence factors of Hp, CagA remains the only one which translocates into cells *via* type IV secretion system (Jimenez-Soto *et al*, 2009; Odenbreit *et al*, 2000; Song *et al*., 2013). For the mechanisms of CagA delivery, recent study revealed that after being exposed on the bacterial surface *via* type IV secretion, CagA interacts with phosphatidylserine of host plasma membrane and then enters the host cells *via* an endocytic process distinct from known endocytic pathways (Murata-Kamiya *et al*., 2010). CagA was also found in serum-derived exosomes in patients with Hp infection, and CagA-containing exosomes can be internalized into cells *via* endocytosis or membrane fusion, independently of type IV secretion (Shimoda *et al*., 2016). Therefore, it is acceptable that, by directly interacting with phosphatidylserine of host plasma membrane, exogenous CagA can enter the cells *via* endocytosis. Actually, similarly to the present study, previous *in vitro* studies showed that recombinant CagA protein may directly influence cell viability or function (Gajewski *et al*., 2015; Lin *et al*., 2015; Wang *et al*., 2016).

CagA can regulate the expression of target proteins in a dose- and/or time-dependent manner (Brandt *et al*., 2005; Kang *et al*., 2013; Lina *et al*., 2013). However, both

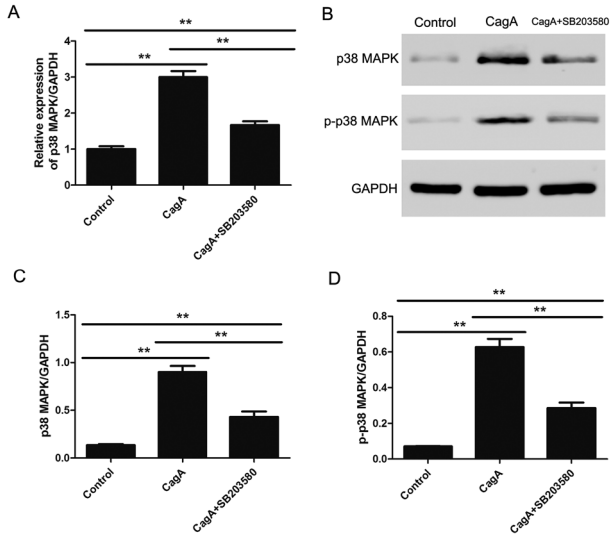


**Figure 4. CagA promoted p38 MAPK expression and phosphorylation in mouse podocytes at 72 h of incubation.**
(**A**) Real time RT-PCR showed that when compared to control, CagA significantly increased the p38 MAPK mRNA level, while pretreatment with SB203580 partly diminished this effect. (**B**) Western blotting analysis revealed that in comparison to control, CagA significantly upregulated p38 MAPK protein expression and phosphorylation (p-p38 MAPK), while pretreatment with SB203580 partly prevented these changes. (**C** and **D**) Quantitative analysis of relative protein level of p38 MAPK (C) and p-p38 MAPK (**D**) (normalized to GAPDH). *$p<0.05$, compared to control; **$p<0.01$, compared to control.

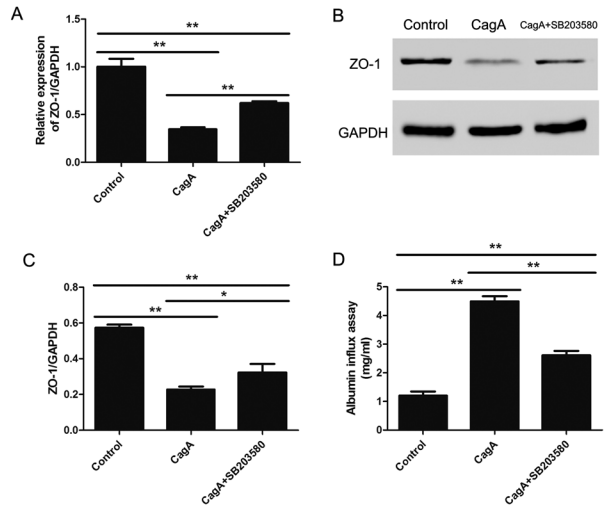


**Figure 5. Selective p38 MAPK inhibition improved ZO-1 expression and filtration barrier function in mouse podocytes treated with CagA for 72h.**
(**A** and **B**) Real time RT-PCR (A) and western blotting analysis (**B**) showed that pretreatment with SB203580 notably increased the mRNA level and protein expression of ZO-1 which was downregulated by CagA treatment. (**C**) Quantitative analysis of relative protein level of ZO-1 (normalized to GAPDH). (**D**) Albumin influx assay revealed that, when compared to CagA treatment, pretreatment with SB203580 significantly reduced albumin influx across the podocyte monolayer. *$p<0.05$, compared to control; **$p<0.01$, compared to control.

unchanged and decreased ZO-1 expression were reported in gastric epithelial cells with CagA-positive Hp infection (Krueger *et al*., 2007; Zhang *et al*., 2014). While CagA-positive Hp infection activated β-catenin signaling in gastric epithelial cells, β-catenin inhibited the expression of ZO-1 in podocytes (Murata-Kamiya, 2011; Zhou *et al*., 2015). Therefore, it seems reasonable that CagA may have the potential to suppress ZO-1 expression *via* β-catenin. In the present study, we found that CagA could dose- and time-dependently decrease ZO-1 expression in mouse podocytes.

Tight junction protein ZO-1 located in the membrane surface of podocytes and playes an essential role in establishing podocyte filtration barrier (Ha, 2013; Itoh *et al*., 2014). Abnormal expression and/or distribution of ZO-1 may impair the filtration barrier function of podocytes and then induce proteinuria. In MWF rats, an animal model of spontaneous proteinuria, abnormal glomerular ZO-1 distribution alone may cause proteinuria (Ha, 2013). In animal model of diabetic kidney disease, high glucose decreases the expression and simultaneously alters the distribution of ZO-1 in glomerular epithelial cells, which is associated with proteinuria (Rincon-Choles *et al*., 2006). In the present study, we demonstrated that after CagA treatment, consistently with the reduced expression of total ZO-1, the distribution of membrane ZO-1 was also decreased, which consequently induced an increased albumin influx across the podocyte monolayer, resulting in an impaired filtration barrier function of mouse podocytes.

The p38 MAPK signaling pathway plays a crucial role in regulating cellular response (Cuadrado & Nebreda, 2010). CagA-positive Hp infection or CagA transfection may induce p38 MAPK phosphorylation in gastric epithelial cells (Allison *et al*., 2009; Liu *et al*., 2012). In Hp-dependent gastric mucosa-associated lymphoid tissue (MALT) lymphoma, the expression level of CagA was closely associated with the activation of p38 MAPK (Kuo *et al*., 2015). In the present study, we also revealed that CagA promoted the expression and phosphorylation of p38 MAPK in mouse podocytes. On the other hand, consistently with the previous reports, SB203580 pretreatment significantly inhibited these changes (Hai-Yan *et al*., 2013; Ding *et al*., 2015).

P38 MAPK activation mediated calcium oxalate crystal-induced downregulation of ZO-1 in distal renal tubular epithelial cells and regulated the distribution of ZO-1 in cell-cell contacts of keratinocytes (Peerapen & Thongboonkerd, 2013; Minakami *et al*., 2015). Both in human podocyte injury diseases and animal models of nephropathy, podocyte p38 MAPK signaling was activated and associated with proteinuria. P38 MAPK inhibition significantly ameliorated podocyte injury and suppressed proteinuria (Koshikawa *et al*., 2005). In the present study, we revealed that p38 MAPK inhibition by SB203580 pretreatment markedly improved CagA-induced downregulation of ZO-1 and consequently protected the filtration barrier function of mouse podocytes.

In summary, our results suggest that CagA, one of the key virulence factors of Hp, decreases the expression and membrane distribution of tight junction protein ZO-1, impairs the filtration barrier function of podocytes, while activating p38 MAPK signaling pathway in these cells. Selective p38 MAPK inhibition partly prevents CagA-induced filtration barrier dysfunction of podocytes through ameliorating ZO-1 downregulation. Therefore, CagA, at least *via* p38 MAPK signaling pathway, may induce podocyte injury. Anti-Hp therapy may be beneficial for the treatment of kidney diseases related to Hp antigen deposition.

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