Helicobacter Pylori UreB Upregulates the Expression of PD-L1 through Myh9 /mTOR Pathway and Inhibits the Activation of CD8+ T Cells

Jian Wu

Wuhan Medical and Health Center for Women and Children: Wuhan Women and Children Medical Care Center

Honghao Wang

Hubei Minzu University: Hubei University for Nationalities

Xia Guo

Wuhan Medical and Health Center for Women and Children: Wuhan Women and Children Medical Care Center

Qinzhen Cai

Wuhan Medical and Health Center for Women and Children: Wuhan Women and Children Medical Care Center

Tian Xiang

Hubei Minzu University: Hubei University for Nationalities

Wei Luo

Tianjin Medical University

Yun Xiang

Wuhan Medical and Health Center for Women and Children: Wuhan Women and Children Medical Care Center

Gao Liu

Hubei Minzu University: Hubei University for Nationalities

Chunhui Yuan (✉ chunhui.yuen@whu.edu.cn)

Wuhan Medical and Health Center for Women and Children, Tongji Medical College, Huazhong University of Science and Technology <https://orcid.org/0000-0003-4382-2541>

Research Article

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Abstract

Objectives: Immune regulation mechanism of how *Helicobacter pylori* urease disrupting the homeostasis of host cells remains unknown.

Methods: We thus detected the effect of *Helicobacter pylori* UreB on macrophage PD-L1 expression with recombinant protein and defective strains. The influence of UreB induced PD-L1 on CD8⁺ T cells' proliferation and perforin and granzyme expression were assessed through co-culture model.

Results: Urease subset B (UreB) significantly promoted PD-L1 expression in Bone marrow-derived macrophages (BMDMs) and thus blocked the proliferation and activity of *H. pylori*-primed CD8⁺ T cells. Myosin heavy chain 9 (Myh9) works as the receptor for UreB. The interaction between UreB and Myh9 promoted amino acid anabolism, activated mTOR pathway and induced PD-L1 expression in BMDMs. mTOR inhibitor Temsirolimus reversed UreB-induced PD-L1 expression and the inhibitory effects on CD8⁺ T cells.

Conclusion: Our study reveals a hitherto-unknown immunosuppressive mechanism of UreB during *H. pylori* infection, provides clues for the development of *H. pylori* vaccine.

Introduction

Gram negative *H. pylori*, which was identified as class-I carcinogen, infected half of the adult population worldwide (Hatakeyama, 2017; Hooi et al., 2017). It's well known that *H. pylori* contributes to the development of chronic gastritis, peptic ulcers and gastric cancer (Uemura et al., 2001). Triple or quadruple antibiotic therapy is the main treatment for *H. pylori* infection. However, with the increase in resistant *H. pylori* strains, the cure rate of antibiotic treatment is decreasing year by year (Savoldi et al., 2018). Even though monovalent or multivalent vaccines based on *H. pylori*'s multiple immune antigens, such as CagA, VacA, NAP, and urease are protective in mammalian model (Zeng et al., 2015; Malfertheiner et al., 2018), but it's regrettable that these vaccines are not effective enough in clinical trials.

Urease, which constitutes around 10% of the total *H. pylori* protein, not only plays a role in acid neutralization, but also disrupts the tight cell junctions, breaches the cellular integrity, and damages the gastric epithelium through producing ammonia (Lytton et al., 2005; Wroblewski et al., 2009). Besides, urease has been reported to promote tumor growth and metastatic dissemination through inducing angiogenesis and it plays a key role in gastric cancer progression (Olivera-Severo et al., 2017). *H. pylori* urease consists of two different subunits of 26.5 kDa (UreA) and 61.7 kDa (UreB) (Ha et al., 2001). Highly conserved UreB is the foremost active component of urease as well as the most promising candidate vaccine antigens (Guo et al., 2019). An oral vaccine based on UreB showed 55% efficacy against *H. pylori* infection in children in a two years' follow-up survey (Zeng et al., 2015). On the other hand, *H. pylori* urease was reported to promote Treg differentiation and asthma protection in neonatally infected mice (Koch et al., 2015). What's importantly, *H. pylori* urease is required for Treg induction and immune tolerance (Koch and Muller, 2015). Furthermore, UreB also induces apoptosis of gastric epithelial cells

through interacting with MHC-II (Fan et al., 2000). Therefore, the limited effectiveness of UreB-based vaccine may be attributed to the immunosuppressive function of UreB.

In this study, we found out that UreB upregulates the expression of PD-L1 through Myh9 /mTOR pathway and inhibits the activity of CD8⁺ T cells during *H. pylori* infection. Except for the reported receptor CD74 in gastric epithelial cells (Beswick et al., 2006), UreB was found to induce PD-L1 expression through interaction with a new receptor Myh9 in macrophages. mTOR, which responds to UreB upregulated amino acid level of macrophages, specially mediated this process. Knocking out UreB, interfering Myh9 or inhibiting mTOR signal pathway hindered UreB induced PD-L1. Co-culture assay manifests that UreB induced PD-L1 arrests the proliferation of CD8⁺ T cells and inhibits the expression of perforin and granzyme B. At last, the inhibition of PD-L1 on CD8⁺ T cells was reversed by mTOR inhibitor Temsirolimus. These results found a new strategy adopted by *H. pylori* to disrupt host cells' functions and suppress immune cells, explained the underlying mechanism through which *H. pylori* promoted the occurrence and development of gastric cancer via virulence factor UreB. This provides a new theoretical basis for the design of UreB-based *H. pylori* vaccine.

Materials And Methods

Recombinant UreB Protein Preparation

E. coli BL21 (DE3) cells were transformed with UreB expression plasmid pET-28a with 6×His-tag. Expression of the recombinant UreB protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside for 12 hours at 25°C. The cell pellets were resuspended in phosphate-buffered saline (PBS) and disrupted by dynamic high-pressure homogenization, and the lysates were clarified by centrifugation (12,000 rpm for 15 min at 4°C). The supernatants were incubated with Ni-agarose pre-equilibrated with 10 mM imidazole solution [25 mM tris-HCl, 200 mM NaCl, and 10 mM imidazole (pH 7.4)]. The recombinant proteins were eluted with 20, 40, 80, and 200 mM imidazole buffer, with 25 mM tris-HCl and 200 mM NaCl (pH 7.4). The purified recombinant proteins were further purified using endotoxin-free purification polymyxin B columns and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting with antibody against His. Protein concentration of UreB was determined by the bicinchoninic acid method.

mRNA-Chip assay

10⁹ BMDMs were infected with WT or UreB KO *H. pylori* at MOI=10 for 24 h or treated with purified UreB protein (1 µg/mL) for 12 h, the total RNA of treated cells was extracted with Trizol and sent to Novogene company for mRNA-chip analysis.

H. pylori strains

The wild-type strain *H. pylori* ATCC43504 used in this study was obtained from the National Institute for Communicable Disease Control and Prevention, Chinese Centers for Disease Control and Prevention (Beijing, China). *UreB*-knockout *H. pylori* (*H. pylori*: Δ UreB) was constructed by our group according to previous reports (Schmalstig et al., 2018). The above strains were cultured on brain-heart infusion medium (10% rabbit blood) under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂) at 37°C.

Cell culture

The murine macrophage cell line RAW264.7 and Human gastric cancer cell lines AGS and MNK8 were all purchased from the China Center for Type Culture Collection and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS). Human THP-1 monocyte cell line was suspended in RPMI 1640 culture medium and distributed to 6 well plates at a density of 10⁶ cells per well and differentiated into macrophages during 24 hours of culture with 20 ng/ml of phorbol 12-myristate 13-acetate (PMA) (37°C; 5% CO₂). THP-1 monocyte cell line-derived macrophages were attached to the plastic surface of the plates and confirmed by light microscopy.

Preparation of Bone marrow-derived macrophages (BMDMs)

Bone marrow cells isolated from the femurs and tibias of BALB/c mice were treated with AKT Lysis Buffer (Beyotime, Shanghai, China) and cultured in DMEM (Gibco, UK) supplemented with 10% FBS (Gibco), 1% Penicillin-Streptomycin Solution, and 50 ng/ml macrophage colony stimulating factor (M-CSF, Peprotech) for 6 days to induce differentiation into BMDM (the adherent cells). The purity of BMDM (F4/80⁺) was determined by flowcytometry (FCM). Amino acid-free medium used for amino acid metabolism test was purchased from Thermo Fisher.

UreB-Ni-NTA bead pull-down assay and MS analysis

The BMDM cell lysates were incubated with 10 µg of the purified recombinant UreB protein with 6×His-tags at 4°C overnight. The reaction mixtures were then incubated with 20 µl of Ni-NTA agarose at 4°C for 4 hours. After intensive washing with washing buffer A [50 mM Hepes (pH 7.5), 300 mM NaCl, 20 mM imidazole, and 0.1 mM PMSF], the proteins that were bound to the agarose were eluted with elution buffer (elution buffer containing 250 mM imidazole). The eluted proteins were analyzed by SDS-PAGE or WB. For SDS-PAGE, the specific bands were excised and subjected to LC-MS (TripleTOF 5600+, SCIEX, USA).

Reverse transcription-quantitative real time PCR (RT-qPCR)

The total RNA of treated cells was extracted with Trizol (Invitrogen Corp, Carlsbad, CA, USA) and reverse-transcribed with a cDNA reverse kit (Toyobo, Osaka, Japan) with Oligo dT primers. The reverse transcribed cDNA was used as a template for qPCR reactions along with SYBR Green Real time PCR Master Mix (Toyobo, Osaka, Japan) and 0.4 μ M forward and reverse primers of PD-L1 (Forward 5'-CCTCCGTAAGTACTGATACAA-3', Reverse 5'-TGCTATACCTGACACCTATAAG-3'), (TSINGKE, Wuhan). The qPCR reactions were run on an ABI Step One Plus (Applied Biosystems) under standard cycling conditions. Relative RNA levels were calculated by the comparative cycle threshold (CT) method ($2^{-\Delta\Delta CT}$ method), where CT represents the amplification cycle number at which the fluorescence generated within a reaction rises above a defined threshold fluorescence and $\Delta\Delta CT$ = experimental groups ($Ct_{\text{Target gene}} - Ct_{\text{GAPDH}}$) - control groups ($Ct_{\text{Target gene}} - Ct_{\text{GAPDH}}$). The mRNA levels of each gene in the experimental groups were then expressed as the fold levels relative to the blank control groups and were calculated by the following formula: $2^{-\Delta\Delta CT}$. Each experimental sample contained three replicate wells.

Western blotting

Cells were harvested and lysed in RIPA buffer supplemented with protease inhibitors (P-8340, Sigma-Aldrich, US). Protein concentrations in cell lysates were measured with the Pierce™ BCA protein assay kit (23225, Thermo Fischer Scientific, US), and the same amount of protein was loaded and separated on a polyacrylamide gel after being denatured at 98°C for 8 mins with SDS loading buffer. Proteins were transferred onto a nitrocellulose membrane which was subsequently blocked for 2 h with a 5% BSA solution (prepared in TBST) and incubated overnight with primary antibodies against PD-L1 (13684, CST, USA), His (66005-1-Ig, Proteintech, China), UreB (ab127916, abcam, UK), OMP (18185-1-AP, Proteintech, China), Myh9 (ab241068, abcam, UK), pPI3K (17366, CST, USA), pAKT (13038, CST, USA), pmTOR(5536, CST, USA), GAPDH (60004-1-Ig, Proteintech, China), pGCN2 (ab68427, abcam, UK), GCN2 (ab134053, abcam, UK), Gli1 (3538, CST, USA), Gli2 (2585, CST, USA). Membranes were then incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies and detected by LumiGlo Reserve™ chemiluminescent substrate kit (54-61-01, Sera care, Life Sciences, MA, US). Protein bands were visualized using the UVP Biospectrum™ 500 imaging system with the VisionWorks® images acquisition and analysis software (Analytik Jena, CA, US).

Magnetic Activated Cell Sorting (MACS) of CD8⁺ T cells

The EasySep™ Mouse CD8⁺ T Cell Isolation Kit (STEMCELL Technologies, Canada) was used to isolate CD8⁺ T cells from splenocytes of BALB/c mice by negative selection. The labeled cells were separated using an EasySep™ magnet, and the desired CD8⁺ T cells were poured into a new tube. The purity of CD8⁺ T cells was >95% as determined by FCM.

Co-culture of macrophages with CD8⁺ T cells

Anti-CD3 and anti-CD28 antibodies are used for the activation of CD8⁺ T cells before assessing the inhibition of treated BMDMs. BMDMs (5×10^5 cells/well) were stimulated with purified UreB protein (1 µg/mL) or infected with WT or UreB defective *H. pylori* strains at MOI=10 for 24 h at 37°C. Then, BMDMs were washed with PBS and co-cultured with CD8⁺ T cells (1×10^6 /well) at the presence of PD-L1 blocking antibody anti-PD-L1 (10 µg/mL) or mTOR inhibitor Temsirolimus (5 µM) for 24 h. The proliferation of CD8⁺ T cells was analyzed by CFSE staining. The expression of perforin and granzyme B in CD8⁺ T cells were detected by FCM with PE anti-mouse Perforin (154306, Biolegend, USA) and FITC anti-human/mouse Granzyme B (515403, Biolegend, USA) respectively.

Amino acid metabolism analysis by LC-MS/MS

BMDMs were stimulated with UreB (1 µg/mL) or solvent for 24 h, cells were washed with cold PBS and collected into 1.5 mL EP tube, add 1 mL cold extraction reagent (70% methanol containing 1 µg/mL 2-chlorophenylalanine as an internal standard), Vortex 1 min. Quickly freeze it in liquid nitrogen for 3 minutes and thaw it on ice for 3 minutes, Vortex 2 minutes, and repeat the above 3 steps for 3 cycles. Samples was centrifuged at 12000 rpm, 4°C for 10 minutes. The supernatant was collected and sent for LC-MS/MS analysis.

Amino acid quantification assay

BMDMs were infected with WT or UreB KO *H. pylori* at MOI=10 for indicated time. The total amino acid levels of treated BMDM were measured using the L-Amino Acid Quantification Kit (MAK002; Sigma-Aldrich, St. Louis, MO).

siRNA transfection assay

To detect p-GCN2 (T898) or PD-L1 expression, BMDM were transfected with siRNA-Myh9 for 24 hours and then treated with 1 µg/mL UreB protein or infected with indicated strains at MOI=10 for different time periods. Electro-transfection systems Lonza Nucleofector (Thermo Fisher, Neon) was used in this transfection assay. siRNA-Myh9 1 (guide 5'-AGUUUUUAUCCACAUAGAGGUTT-3', passenger 5'-CUCUAUGUGGAUAAAAACUUCTT -3'), siRNA-Myh9 2 (guide 5'- UGAUGAAGUUUUUAUCCACAUTT-3', passenger 5'- GUGGAUAAAAACUUCAAAUTT -3'), (TSINGKE, Wuhan).

Graphs and Statistical Analysis

Results were plotted using GraphPad Prism v8 and analyzed using an unpaired, two-tailed *t*-test or two-way ANOVA as relevant, with *p*-values represented as *P* > 0.05 ns, **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

Results

UreB upregulates the PD-L1 expression of host cells

H. pylori colonization results in a local infiltration of macrophages, which play a critical role in the gastric complication (Menaker et al., 2004). Here we infected BMDMs with WT or UreB KO *H. pylori* which was validated by western blot and urease activity assay (Figure S1A, S1B) at MOI=10 for 24 h to find out differently expressed genes related to the immune regulating functions of macrophages through mRNA-chip sequencing. As shown in Figure 1A, compared with UreB KO *H. pylori* infected BMDMs, PD-L1 as well as other immune or inflammatory factors of *H. pylori* group were upregulated. Then we verified this phenomenon as *H. pylori* infection induced PD-L1 expression was restrained by defecting UreB (Figure 1B-D). Then we expressed and purified the recombinant UreB protein with His tag which was confirmed by Coomassie brilliant blue staining (Figure S1C) and western blot with anti-His antibody (Figure S1D). The optimal concentration for UreB protein to induce the expression of PD-L1 in BMDMs was confirmed as 1 µg/mL (Figure S1E). The PD-L1 expression of UreB protein (1 µg/mL) treated BMDMs were also upregulated with a time gradient dependence (Figure 1E-G). Besides, UreB induced PD-L1 expression is not limited to Human or murine macrophages as we found UreB also upregulated the PD-L1 expression of gastric cancer cell lines such as AGS and MNK28 (Figure S1F). These results indicated that UreB contributes to *H. pylori* induced PD-L1 expression in host cells.

UreB dampens the activation of CD8⁺ T cells through the induction of macrophage PD-L1 expression

As it has been reported that UreB could be found inside macrophages (Schwartz and Allen, 2006), so, *H. pylori* may become a kind of intracellular bacteria (Kronsteiner et al., 2014). Since *H. pylori*-induced PD-L1 can inhibit the proliferation of activated T-cells (Beswick et al., 2007). We use UreB treated BMDMs to co-culture with CFSE stained CD8⁺ T cells which were isolated from the spleen of wild type BALB/c. Then the proliferation ability of CD8⁺ T cells was measured with FCM. As shown in (Figure 2A, S2A), UreB treated BMDMs inhibited the proliferation of CD8⁺ T cells, however, when PD-L1 was blocked by PD-L1 blocking antibody, the inhibition effect was partly reversed, suggesting that UreB induced PD-L1 mediated the inhibition on CD8⁺ T cells proliferation. Next, we get the consistent results from *H. pylori*-infection assay. WT *H. pylori* infected macrophages inhibited the proliferation of CD8⁺ T cells, but when UreB was knocked out, the inhibition effect was blocked. In addition, when PD-L1 was neutralized with PD-L1 antibody, the proliferation index of both WT and UreB KO group increased (Figure 2B, S2B). Except for the influence on CD8⁺ T cells' proliferation, the cytotoxic function of CD8⁺ T cells was detected with FCM in the same co-culture model. Compared with the control group (CD8⁺ T cells only), UreB treated (Figure 2C, D) or WT *H. pylori* infected (Figure 2E, F) BMDMs have a stronger inhibition on the perforin and granzyme B expression of CD8⁺ T cells, and PD-L1 blocking antibody eliminate this change. These results manifest that UreB induced PD-L1 is critical for the suppression of macrophages on CD8⁺ T cells.

UreB interacted with Myh9 to upregulate the expression of PD-L1

To explore the exact mechanism through which UreB upregulated the expression of PD-L1, we use UreB-his-Ni-agarose to pulldown the receptor for UreB in BMDMs. As it has been reported that UreB increases MLC (myosin II regulatory light chain) phosphorylation, occludin internalization and barrier dysfunction in gastric epithelial cells during *H. pylori* infection (Wroblewski et al., 2009). We selected two potential targets Hspe1 and Myosin-9 (Myh9) from all detected proteins in LC-MS (Figure 3A, S3A-B). When we knock down these two genes with siRNAs respectively, interfering Myh9 rather than Hspe1 blocked UreB-induced PD-L1 expression (Figure 3B). And then the interaction between UreB and Myh9 during UreB stimulation was further confirmed by co-immunoprecipitation with antibody against His (Figure 3C) or Myh9 (Figure 3D), respectively. When BMDMs were infected with WT or UreB KO *H. pylori*, Myh9 immunoprecipitated UreB in WT *H. pylori*-infected BMDMs (Figure 3E), but not UreB KO group. In addition, the interference of Myh9 expression also downregulated *H. pylori* induced PD-L1 expression of BMDMs, but this effect disappeared when BMDMs were infected with UreB KO strain (Figure 3F). Taken together, these results suggested that Myh9 works as a receptor for UreB and mediates the upregulation of PD-L1 in *H. pylori*-infected BMDMs.

mTOR signal pathway mediated UreB induced PD-L1 expression

To further evaluate the effect of UreB on host cells' signal pathway, we make a gene set enrichment analysis on UreB induced differently expressed genes. As shown in Figure 4A, the most enriched genes are related to amino acids metabolism (about 300 genes). And, there are about 100 genes associated with mTOR signal pathway. It is consistent with the report that c-Met/AKT/mTOR pathway activated by MACC1 (metastasis associated in colon cancer-1) contributes to the PD-L1 expression in gastric cancer cells and induces tumor growth in vivo (Tong et al., 2019). We found that *H. pylori* contributes to the activation of mTOR as well as the PD-L1 expression during *H. pylori* infection whereas PD-L1 were downregulated when UreB was depleted (Figure S3C). Therefore, we further examined if mTOR is associated with UreB induced PD-L1 expression. BMDMs, pretreated with inhibitors for PI3K (LY294002), AKT (Ipatasertib), mTOR (Temsirolimus) and Hedgehog/Gli (GANT61) for 1 h, were then infected with *H. pylori* at MOI=10 for 3 or 12 h, and the mRNA level of PD-L1 was detected with RT-qPCR. Temsirolimus and GANT61 instead of LY294002 and Ipatasertib arrest *H. pylori* induced PD-L1 expression (Figure 4B-E). These results mean that mTOR mediated UreB induced PD-L1 just like CagA induced PD-L1 expression through sonic Hedgehog (Shh) signaling pathway (Holokai et al., 2019). Then, BMDMs were pretreated with inhibitors against PI3K/AKT/mTOR, and then stimulated with purified UreB protein (1 µg/mL) for 1 or 3 h, and the activity of PI3K/AKT/mTOR signaling pathway was analyzed by immune blots. Even through UreB activated PI3K/AKT/mTOR axis, but only mTOR inhibitor Temsirolimus blocked UreB induced PD-L1 (Figure 4F), which indicates that UreB activated mTOR is independent of PI3K/AKT.

Then it was confirmed that UreB induced PD-L1 expression during *H. pylori* infection is independent of shh signaling pathway which mediated CagA upregulated PD-L1 (Figure 4G). Besides, when the receptor of UreB was interfered with verified effective siRNA-Myh9 2 (Figure S3D), UreB activated mTOR was also weakened (Figure 4H). These data reach a conclusion that UreB activated mTOR leads to the upregulation of PD-L1 on macrophages.

mTOR response to UreB disrupted amino acid metabolism

As UreB activated mTOR is independent of PI3K/AKT, and genes about amino acids metabolism are enriched in UreB stimulated BMDMs (Figure 4A). We wonder if UreB disrupts the amino acids metabolism of host cells to activate mTOR, so the relative content of all detected L-amino acids by LC-MS/MS analysis were shown in Figure 5A. It is obvious that most detected L-amino acids increased after being stimulated with UreB. And we get a consistent result when the amino acids level of WT or UreB KO *H. pylori* infected BMDMs were detected with L-Amino Acid Quantitation Kit. It's clear that even though *H. pylori* infection disrupted the amino acids metabolism of host cells which showed a decrease in amino acids level, UreB reversed the amino acids exhaustion (Figure 5B). To directly evaluate the influence of UreB on amino acids pool, the autophosphorylation state at Thr 898 of GCN2 (p-GCN2 (T898)) was examined, which increases as a consumed marker of available pools of amino acids (Dever and Hinnebusch, 2005). Compared to the WT *H. pylori* infected BMDMs, UreB KO group upregulated the expression of phosphorylated GCN2 (p-GCN2 (T898)) (Figure 5C). However, when UreB receptor was disturbed with siRNA, there was no difference between the WT and UreB KO group (Figure 5D). It is worth noting that despite Myh9 was disabled, WT and UreB KO *H. pylori* can still induce the expression of p-GCN2 (T898) (Figure 5D), which means that there are some other potential virulence effectors such as CagA contribute to the depletion of amino acids in host cells (Kim et al., 2018). And then our previous hypothesis was confirmed as when BMDMs were cultured in amino acids free medium, neither WT or UreB KO *H. pylori* could active mTOR signaling pathway and induce the expression of PD-L1 (Figure 5E). These results unraveled that UreB activated mTOR pathway triggered amino acid anabolism, leading to the induction of PD-L1 expression.

mTOR inhibitor blocked the inhibition of UreB up-regulated PD-L1 on CD8⁺ T cells

Given that the above results have confirmed that mTOR mediated the expression of UreB induced PD-L1. It's of particular interest that whether mTOR is involved in the inhibitory effect of PD-L1 on CD8⁺ T cells. UreB treated BMDMs were cocultured with CD8⁺ T cells at the presence of Temsirolimus. As inhibited mTOR limited the expression of PD-L1, the overall level of proliferation (Figure 6A, S2C) as well as the secretion of cytotoxic factors perforin and granzyme B (Figure 6B) are higher than the control groups (DMSO treated). And the inhibition of *H. pylori* infected macrophages on CD8⁺ T cells was also blocked by Temsirolimus (Figure 6CD, S2D). However, the blocking effect of Temsirolimus vanished when UreB

was KO (Figure 6C). It's interesting that Temsirolimus could not completely reverse the influence of *H. pylori* infected macrophages on the production of cytotoxic factors in CD8⁺ T cells, which may be caused by other virulence factors (except for UreB) that could inhibit CD8⁺ T cells' function independent of mTOR signaling pathway (Figure 6D). The above results suggest that UreB activated mTOR signaling pathway is critical for the inhibition of macrophages on CD8⁺ T cells during *H. pylori* infection.

Conclusion

Overall, we found out that UreB upregulates the PD-L1 expression of macrophages through Myh9 /mTOR pathway and inhibits the activity of CD8⁺ T cells during *H. pylori* infection. UreB was found to induce PD-L1 expression through interaction with a new receptor Myh9 in macrophages. mTOR, which responds to UreB upregulated amino acid level of macrophages, specially mediated this process. UreB induced PD-L1 arrests the proliferation of CD8⁺ T cells and inhibits the expression of perforin and granzyme B. In light of these results, we demonstrated the immunosuppressive function of UreB on host cells.

Discussion

To explore the relationship between UreB induced immunosuppressive molecule PD-L1 and CD8⁺ T cells, we focused on the mechanism research which clarified how UreB upregulated PD-L1. However, there are so many potential genes that were upregulated by UreB in Fig. 1A. For example, UreB contributes to the expression of many kinds of chemokines and TNF, suggesting that UreB participated in the induction of inflammatory response against *H. pylori* during infection.

Consistent with the report that CagA induced PD-L1 expression within the gastric epithelium via Shh signaling pathway during *H. pylori* infection (Holokai et al., 2019), UreB defective *H. pylori* could still induce the expression of PD-L1 which is independent of Myh9. It's possible that there are some additional virulence factors that contribute to the expression of PD-L1. As blockade of the programmed cell death 1(PD-1)/PD-1 ligand-1(PD-L1) pathway has been reported to reverses dysfunction of CD8⁺ T cells in liver cancer and ovarian cancer (Wang et al., 2019; Qian et al., 2020), it is possible that UreB induced PD-L1 directly contract CD8⁺ T cells' PD1 to exert immunosuppressive effect.

A direct interaction between Hsp60 and the N terminal of UreA has been reported in *H. pylori* (Zhao et al., 2019). Even though Hspe1 was identified as a potential receptor for UreB by LC-MS, but no difference in UreB induced PD-L1 was found when Hspe1 was interfered with siRNA, which indicated that UreB may influence other signaling pathway except for PD-L1 through Hspe1. At least, we found a direct interaction between UreB and the heat shock protein family of host cells, so a further research focused on HSP mediated pathogenic mechanism during *H. pylori* infection will be valuable.

mTOR was reported to regulate the expression of PD-L1 in gastric cancer (Tong et al., 2019), vice versa, PD-L1 promotes the proliferation of HNSCC cells through mTOR (Zheng et al., 2019). UreB upregulated immunosuppressive molecular PD-L1 through mTOR but not PI3K/AKT. Meanwhile, to re-establish

metabolic homeostasis, mTOR coordinates cellular process in response to nutrient or energy stress (Deretic and Levine, 2009; Kroemer et al., 2010). It's a novel discovery that mTOR response to the amino acids level of host cells which connect the immunosuppressive phenomenon with host amino acids metabolism. But we only detected the mTOR activation in response to amino acids, and it will also be important to examine if UreB influences the glucose metabolism, as it has been reported that *H. pylori* CagA upregulates glucose metabolism of gastric cancer cells (Gao et al., 2020). And over expressed PD-L1 accelerates glycolysis by AKT/mTOR/HIF-1 α axis in acute myeloid leukemia (Ma et al., 2020). As UreB promoted amino acid anabolism indicates a rich nutrients and energy state which is conducive to proliferation and tumorigenesis. Except for regulating PD-L1, UreB activated PI3K/AKT may influence the survival and proliferation of host cells (Xu et al., 2018; Yao et al., 2020). As to pathogen, UreB upregulated the amino acids synthesis and provided an environment with enough energy and nutrients for *H. pylori* survival. It is possible that UreB may inhibit the autophagy of host cells because CagA activated mTOR inhibits the autophagy of AGS cells (Li et al., 2017).

Except for directly damaging host cells, pathogens can also indirectly retain the activity of T cells through host cells to escape protective host immune responses. For example, *M. tuberculosis* infection induced ERK pathway signaling of macrophages suppresses Th1 responses during antigen-specific T cell activation (Richardson et al., 2015). In the same way, *H. pylori* induced PD-L1 contributes the persistence infection and progression of disease to cancer by shutting down T cell effector function (Holokai et al., 2019). Our study provides evidence that UreB inhibits the function of CD8⁺ T cells through it induced PD-L1 of host cells. However, it is a pity that we can't establish a *H. pylori* infection mouse model to verify the influence of UreB on PD-L1 CD8⁺ T cells. It's no doubt that *H. pylori* infection contributes to the progression of gastric cancer. So, there is a need for a substantial effort ahead to directly demonstrate if the inhibition of UreB induced PD-L1 on CD8⁺ T cells exacerbates the development of *H. pylori* related gastric cancer.

In conclusion, our findings firstly demonstrated that *H. pylori* UreB upregulated PD-L1 through activated mTOR, then PD-L1 inhibited the proliferation and activity of CD8⁺ T cells. Here, we successfully identified Myh9 as a new receptor for UreB and mediated the regulation on PD-L1 as well as CD8⁺ T cells. UreB disrupted amino acids metabolism is involved with the suppression on CD8⁺ T cells, which revealed that *H. pylori* inhibits the immune response by disrupting the host metabolism (Fig. 7). In light of our results, it is reasonable that taking the immunosuppressive effect into consideration when developing UreB based *H. pylori* vaccine.

Declarations

Ethics statement

All mouse experiments in this study were reviewed and approved by the ethics committee of Tianjin Medical University General Hospital.

Availability of supporting data

All datasets generated for this study are included in the manuscript/Supplementary Files.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

Chunhui Yuan and Gao Liu designed the research. Jian Wu, Honghao Wang and Xia Guo conducted the experiments. Qinzhen Cai and Tian Xiang analyzed the data. Yun Xiang supervised the research. Chunhui Yuan and Jian Wu wrote the manuscript. Wei Luo revised the manuscript.

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Authors' information

1. Jian Wu: JianWu@whu.edu.cn
2. Honghao Wang: wanghh201812@163.com
3. Xia Guo: 364042161@qq.com
4. Qinzhen Cai: cai200504046@126.com
5. Tian Xiang: kinki-snow@whu.edu.cn
6. Wei Luo: weiluo1028@sina.com
7. Yun Xiang: xiangyun5272008@163.com
8. Gao Liu: lgkinki@whu.edu.cn
9. Chunhui Yuan: yuen@whu.edu.cn

Consent for publication

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Figures

Figure 1

UreB upregulates the expression of PD-L1. (A) BMDMs were infected with WT or UreB KO *H. pylori* at MOI=10 for 24 h, cells were collected and sent for mRNA-chip analysis, differently expressed genes related to macrophages functions were shown. (B-D) BMDMs were infected with WT or UreB KO *H. pylori* at MOI=10 for the indicated time, then PD-L1 expression were detected by RT-qPCR (B), western blotting (C) and FCM (D), respectively. Two-way ANOVA with Bonferroni's multiple comparison test versus *H.*

pylori was used in (B). (E-G) BMDMs were treated with UreB (1 $\mu\text{g/mL}$) for the indicated time, then the expression of PD-L1 was analyzed by RT-qPCR (E), western blotting (F), and FCM (G), respectively. Unpaired t test, versus 0 hours was used for analysis in (E). The data are expressed as the mean $\pm$ SEM, n = 3; NS ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$.

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Figure 2

UreB treated BMDMs inhibited the proliferation and activity of CD8⁺ T cells. (A, B) BMDMs were induced into M0 with M-CSF (50 ng/mL) for 3 days. Then it was treated with purified UreB (A) or infected with indicated *H. pylori* strains (B) at MOI=10 for 24 h, then BMDMs were washed with PBS 3 times and co-cultured with CFSE stained CD8⁺ T cells in the presence of PD-L1 blocking antibody for 24 h. The proliferation of CD8⁺ T cells was analyzed by Flow cytometry. 20000 CD8 positive events were collected. Unpaired t test versus *H. pylori* was used for analysis. (C, E) UreB treated BMDMs (C) or indicated *H. pylori* strains infected BMDMs (E) were co-cultured with CD8⁺ T cells in the absence or presence of blocking antibody against PD-L1 for 24 h, then perforin and granzyme B of CD8⁺ T cells were detected by FCM. The control group represents only CD8⁺ T cells. (D, F) Percentage of perforin or granzyme B positive CD8⁺ T cells in (C) and (E) were presented, respectively. Unpaired t test versus UreB (D) or *H. pylori* (F) were used for analysis. The data are expressed as the mean $\pm$ SEM, n = 3; NS (P > 0.05), **P < 0.01, ***P < 0.001.

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Figure 3

UreB interacted with Myh9 to upregulate PD-L1. (A) The BMDM cell lysates were incubated with 10 μ g of the purified recombinant UreB protein with 6 $\times$ His-tags at 4 $^{\circ}$ C overnight. The reaction mixtures were then incubated with 20 μ l of Ni-NTA agarose at 4 $^{\circ}$ C for 4 hours. Ni-agarose captured complex were identified by LC-MS, the captured peptides sequence of the Hspe1 and Myh9 which got a high scores were shown. (B) 24 hours after being electro-transfected with siRNA specific for Myh9 or Hspe1, BMDMs were treated with UreB protein (1 μ g/mL) for indicated time, then the mRNA level of PD-L1 were detected by RT-qPCR. Unpaired t test versus control group was used for analysis. (C, D) BMDMs were stimulated with purified

UreB for 15 and 30 min or without, then cells were collected and immunoprecipitation was performed with antibodies anti-His (C) or anti-Myh9 (D), immunoblots was performed to detect UreB and Myh9. (E) BMDMs were infected with indicated strains for 30 mins, then co-IP was performed with antibody anti-Myh9 and immunoblots was performed to detect UreB and Myh9. (F) BMDMs were infected with indicated strains for 24 h after being electro-transfected with siRNA-Myh9 or siRNA-control, the mRNA level of PD-L1 was detected by RT-qPCR. Unpaired t test Myh9 KD versus control was used for analysis. The data are expressed as the mean $\pm$ SEM, n = 3; NS (P > 0.05), *P < 0.05, **P < 0.01.

Figure 3

UreB interacted with Myh9 to upregulate PD-L1. (A) The BMDM cell lysates were incubated with 10 µg of the purified recombinant UreB protein with 6×His-tags at 4°C overnight. The reaction mixtures were then incubated with 20 µl of Ni-NTA agarose at 4°C for 4 hours. Ni-agarose captured complex were identified by LC-MS, the captured peptides sequence of the Hspe1 and Myh9 which got a high scores were shown. (B) 24 hours after being electro-transfected with siRNA specific for Myh9 or Hspe1, BMDMs were treated with UreB protein (1 µg/mL) for indicated time, then the mRNA level of PD-L1 were detected by RT-qPCR. Unpaired t test versus control group was used for analysis. (C, D) BMDMs were stimulated with purified UreB for 15 and 30 min or without, then cells were collected and immunoprecipitation was performed with antibodies anti-His (C) or anti-Myh9 (D), immunoblots was performed to detect UreB and Myh9. (E) BMDMs were infected with indicated strains for 30 mins, then co-IP was performed with antibody anti-Myh9 and immunoblots was performed to detect UreB and Myh9. (F) BMDMs were infected with indicated strains for 24 h after being electro-transfected with siRNA-Myh9 or siRNA-control, the mRNA level of PD-L1 was detected by RT-qPCR. Unpaired t test Myh9 KD versus control was used for analysis. The data are expressed as the mean ± SEM, n = 3; NS (P > 0.05), *P < 0.05, **P < 0.01.

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UreB interacted with Myh9 to upregulate PD-L1. (A) The BMDM cell lysates were incubated with 10 μ g of the purified recombinant UreB protein with 6 $\times$ His-tags at 4°C overnight. The reaction mixtures were then incubated with 20 μ l of Ni-NTA agarose at 4°C for 4 hours. Ni-agarose captured complex were identified by LC-MS, the captured peptides sequence of the Hspe1 and Myh9 which got a high scores were shown. (B) 24 hours after being electro-transfected with siRNA specific for Myh9 or Hspe1, BMDMs were treated with UreB protein (1 μ g/mL) for indicated time, then the mRNA level of PD-L1 were detected by RT-qPCR. Unpaired t test versus control group was used for analysis. (C, D) BMDMs were stimulated with purified

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Figure 4

UreB upregulates PD-L1 dependent on activated mTOR. (A) BMDMs were treated with or without purified UreB protein (1 $\mu\text{g/mL}$) for 12 h and cells were collected and sent for mRNA-chip analysis. UreB induced differentially expressed signaling pathways are displayed. (B-E) BMDMs were pretreated with inhibitors of PI3K (LY294002), AKT (Ipatasertib), mTOR (Temsilolimus) and Hedgehog/Gli (GANT61) for 1 h, then it was infected with *H. pylori* at MOI=10 for 0, 3, 12 h, then the mRNA of PD-L1 was analyzed by RT-qPCR. Unpaired t test versus control was used for analysis. (F) Indicated inhibitors pretreated BMDMs were simulated with UreB (1 $\mu\text{g/mL}$) for indicated time, then the protein level of PD-L1 was analyzed by immune blots. (G) The PD-L1 expression of BMDMs which were infected with indicated strains with the presence of Hedgehog/Gli inhibitor (GANT61) was detected by immune blots, and the inhibitory effect of GANT61 was shown with the protein level of Gli1 and Gli2. (H) BMDMs were electro-transfected with siRNA-Myh9 and then stimulated with UreB (1 $\mu\text{g/mL}$) for indicated time, and the PD-L1 as well as mTOR expression was analyzed by western blots. The data are expressed as the mean $\pm$ SEM, n = 3; NS (P > 0.05), *P < 0.05, **P < 0.01, or ***P < 0.001.

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Figure 5

UreB upregulates the amino acids levels via Myh9/mTOR pathway. (A) BMDMs were stimulated with UreB (1 $\mu\text{g/mL}$) or solvent for 24 h, then cells were collected and sent for LC-MS/MS analysis. The difference of all detected L-amino acids was shown. (B) The amino acids level of WT or UreB KO *H. pylori* infected (MOI=10) BMDMs were detected with L-Amino Acid Quantitation Kit. Unpaired t test versus *H. pylori* was used for analysis. (C) BMDMs were infected with indicated strains at MOI=10 for 24 h, the total or phosphorylated GCN2 (T898) were analyzed by western bolts. (D) BMDMs were electro-transfected with siRNAs Myh9, then it was infected with indicated strains at MOI=10 for 24 h, GCN2 and p-GCN2 (T898) were detected by western blots. (E) BMDMs cultured in amino acids free or complete medium

were infected with indicated strains at MOI=10 for 24 h, then the expression of PD-L1 and mTOR were analyzed with immunoblots. The data are expressed as the mean $\pm$ SEM, n = 3; NS (P > 0.05), *P < 0.05, ***P < 0.001.

Figure 5

UreB upregulates the amino acids levels via Myh9/mTOR pathway. (A) BMDMs were stimulated with UreB (1 μ g/mL) or solvent for 24 h, then cells were collected and sent for LC-MS/MS analysis. The difference of all detected L-amino acids was shown. (B) The amino acids level of WT or UreB KO *H. pylori* infected (MOI=10) BMDMs were detected with L-Amino Acid Quantitation Kit. Unpaired t test versus *H. pylori* was used for analysis. (C) BMDMs were infected with indicated strains at MOI=10 for 24 h, the total or phosphorylated GCN2 (T898) were analyzed by western bolts. (D) BMDMs were electro-transfected with siRNAs Myh9, then it was infected with indicated strains at MOI=10 for 24 h, GCN2 and p-GCN2 (T898) were detected by western blots. (E) BMDMs cultured in amino acids free or complete medium were infected with indicated strains at MOI=10 for 24 h, then the expression of PD-L1 and mTOR were analyzed with immunoblots. The data are expressed as the mean $\pm$ SEM, n = 3; NS (P > 0.05), *P < 0.05, ***P < 0.001.

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Figure 6

mTOR mediated UreB induced inhibition on CD8⁺ T cells. (A) BMDMs were treated with purified UreB (1 μ g/mL) (A) or infected with indicated *H. pylori* strains (C) at MOI=10 for 24 h, then BMDMs were washed with PBS 3 times and co-cultured with CFSE stained CD8⁺ T cells at the presences of mTOR inhibitor Tamsirolimus for 24 h. The proliferation of CD8⁺ T cells was analyzed by Flow cytometry. (B, D) UreB (1 μ g/mL) stimulated (B) or indicated strains infected (D) (MOI=10) BMDMs which have been pretreated

with mTOR inhibitor Temsirolimus were co-cultured with CD8+ T cells for 24 h, then perforin and granzyme B of CD8+ T cells were detected by FCM. The statistical histograms were shown at the right. Unpaired t test versus DMSO (B) or *H. pylori* (D) were used for analysis. The data are expressed as the mean $\pm$ SEM, n = 3; NS (P > 0.05), *P < 0.05, ***P < 0.001.

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Figure 7

Illustration of the effects of UreB on macrophages and CD8+ T cells during H. pylori infection.

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