Interleukin 10 suppresses lysosome-mediated killing of Brucella abortus in cultured macrophages

Huynh Tan Hop¹, Alisha Wehdnesday Bernardo Reyes¹, Tran Xuan Ngoc Huy¹, Lauren Togonon Arayan¹, WonGi Min¹, Hu Jang Lee¹, Man Hee Rhee², Hong Hee Chang³, Suk Kim¹,³

¹) Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Jinju 52828, Republic of Korea, ²) College of Veterinary Medicine, Kyungpook National University, Daegu 41566, Republic of Korea, ³) Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea

Running title: Functional characterization of IL10 in B. abortus infection

To whom correspondence should be addressed: Suk Kim, DVM, PhD. Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Jinju 52828, Republic of Korea. Tel: +82-55-772-2359; Fax: +82-55-772-2349. Email address: kimsuk@gnu.ac.kr

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ABSTRACT

Brucella abortus is a Gram-negative zoonotic pathogen for which there is no 100% effective vaccine. Phagosomes in B. abortus-infected cells fail to mature allowing the pathogen to survive and proliferate. Interleukin 10 (IL-10) promotes B. abortus persistence in macrophages by mechanisms that are not fully understood. In this study, we investigated the regulatory role of IL-10 in the immune response to B. abortus infection. B. abortus-infected macrophages were treated with either IL10 siRNA or recombinant IL-10 (rIL-10) and the expression of phagolysosome- or inflammation-related genes was evaluated by qRT-PCR and Western blot. Phagolysosome fusion was monitored by fluorescence microscopy. We found that the synthesis of several membrane-trafficking regulators and lysosomal enzymes was suppressed by IL-10 during infection, resulting in a significant increase in the recruitment of hydrolytic enzymes by Brucella-containing phagosomes (BCPs) when IL-10 signaling was blocked. Moreover, blocking IL-10 signaling also enhanced proinflammatory cytokine production. Finally, concomitant treatment with Stat3 siRNA significantly reduced the suppression of pro-inflammatory brucellacidal activity but not phagolysosome fusion by rIL-10. Thus, our data are first evidences that clearly indicate the suppressive role of IL-10 on phagolysosome fusion and inflammation in responses to B. abortus infection through two distinct mechanisms Stat3-independent and -dependent pathways, respectively, in murine macrophages.

INTRODUCTION

Brucella spp. are facultative intracellular Gram-negative bacteria that cause brucellosis in a variety of mammalian hosts, particularly, they cause more than 500,000 new human cases reported annually (1). They can prevent phagosome maturation by not fully understood mechanisms, leading to successful survival within professional and non-professional phagocytes (2, 3). Vaccination seems to be a predominant manner for the control of infectious diseases; however, there is no 100% efficacious vaccine for brucellosis so far. Thus, identification of host defense mechanisms is essential to design rational approaches to eliminate brucellosis (4).
Interleukin 10 (IL-10) is a pleiotropic immunomodulatory cytokine that is mainly produced by activated Th2 cells, monocytes, macrophages and B cells. It was reported earlier to be a key inhibitor of inflammation and Th1-dependent cell-mediated immunity, especially, production of pro-inflammatory cytokines such as interleukin 1β (IL-1β), tumor necrosis factor (TNF), interleukin 6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokines such as macrophage inflammatory peptide 1α (MIP-1α), generation of nitric oxide (NO) and up-regulation of surface antigen expression (MHC class II, CD80, CD86) in LPS-activated macrophages (5, 6).

Additionally, various studies have also shown that IL-10 plays a suppressive role in host response to Brucella abortus when the survival of intracellular Brucella abortus was markedly decreased in the absence of IL-10 (7, 8). The recent study indicated that IL-10 is beneficial for avoiding the phagolysosome fusion of intracellular Brucella, resulting in prolonged persistence of Brucella in macrophages (9). However, the underlying mechanisms into how IL-10 could suppress the lysosome-mediated killing of Brucella in host cells still need to be clarified.

Phagolysosome fusion with the implication of hundreds of proteins is the most crucial effector in host responses against bacterial infection. Up to date, lysosomal membrane glycoprotein 1, 2 (LAMP-1, LAMP-2) and member RAS oncogene family (RABs) were the only known regulators to be mostly important in controlling this process (10-12). Thus, our effort here is to identify which regulators are controlled by IL-10 during infection. Furthermore, expression of different hydrolytic enzymes was also evaluated since sufficient recruitment of lysosomal enzymes is essential to restrict bacteria within phagosomes. Our findings revealed that IL-10 inhibits lysosome-mediated killing through governing a variety of important proteins such as RAB family, LAMP-1, LAMP-2 and cathepsin (Cts) family by Stat3-independent pathway in B. abortus-infected macrophages.

**RESULTS**

IL-10 represses brucellacidal activity through promoting bacterial ability to avoid late endosomes in macrophages

To monitor the impact of B. abortus infection on IL-10 expression, we first used RT-PCR to evaluate the transcriptional profile of Il10 mRNA in macrophages at different time points of infection. Interestingly, as shown in Fig. 1A, B. abortus infection markedly induced the transcription of Il10 of approximately 2.2, 1.8 and 4.5-fold at 4, 24 and 48h post-infection, respectively, as compared to uninfected cells. Additionally, the production of IL-10 protein measured by indirect ELISA was also consistently obtained with a marked increase of intracellular and secreted IL-10 after infection (Fig. 1B).

To investigate the roles of IL-10 in the brucellacidal activity, RAW 264.7 was pretreated with IL-10 siRNA prior to infection with B. abortus. As shown in Fig. 1C, inhibition of IL-10 signaling caused a significant decrease of intracellular Brucella survival which was in parallel with the observation of colocalization of LAMP-1 and BCPs. In the IL-10 blocking cells, colocalization was approximately 1.4 times higher as compared with control cells (Fig. 1D, E). Taken together, these data clearly indicated that IL-10 prevents the recruitment of lysosomes by BCPs, leading to the persistence of bacteria within macrophages.

Interference of IL-10 signaling alters normal acquisition of membrane-trafficking regulators by B. abortus phagosomes

Fusion of phagosomes with late endocytic organelles (lysosomes) is a central effector of antimicrobial immunity that requires the implication of hundreds of proteins, and this process is suppressed by IL-10, thus identification of regulators mediated by IL-10 could provide insights into its underlying mechanism during Brucella infection. Based from previous reports on phagolysosome regulation (12, 13) and our previous study on microarray analysis of gene expression profiling of B. abortus-infected macrophages (unpublished data), 30 interested trafficking regulators were selected. These transcripts were then assessed by RT-PCR in cells with or without treatment with IL10 siRNA at
different phases of infection. As shown in Fig. 2A, inhibition of IL-10 signaling caused the induction of Lamp1, Lamp2 and Rab34 mRNA at early phase, however, no influence of IL-10 on these genes was obtained at 24h post-infection (Fig. 2B). Interestingly, transcripts of Lamp1, Lamp2, Rab5a, Rab7, Rab20, Rab22a, Rab34, and Stx11 showed to be remarkably increased in IL-10-deficient cells in comparison with the controls at late infection (Fig. 2C).

To complement these data, we checked the expression of selected proteins by Western blot at 4h and 48h post-infection. As expected, the expression of LAMP-2, Rab34 and Rab22A proteins was shown to be consistent with RT-PCR results when blocking of IL-10 signaling significantly increased these proteins compared with the control (Fig. 2D). Furthermore, evaluation of acquisition of these trafficking regulators by Brucella phagosomes at 48 h post-infection logistically revealed that inhibition of IL-10 in macrophages significantly induced the fraction of Brucella phagosomes labeled for LAMP-2 (Fig. 3A, B) and Rab22A (Fig. 3C, D) at late infection.

Altogether, our findings suggest that suppression of IL-10 on phagolysosome fusion might be through inhibition of Lamp-1, Lamp-2, Rab5a, Rab7, Rab20, Rab22A, Rab34, and Stx11 during Brucella infection.

IL-10 mediates the expression and recruitment of hydrolytic enzymes during Brucella infection in macrophages

The acquisition of acidic lysosomal enzymes by pathogen-containing phagosome and their activation result in an efficient killing of intracellular pathogens (12, 14). Thus, we hypothesized that IL-10 also manipulates the expression of these hydrolytic enzymes. To address this hypothesis, the transcriptional profiling of 30 lysosomal enzymes was initially assessed by RT-PCR at different times. Intriguingly, the expression of all enzymes was independent with the deficiency of IL-10 at early and middle phases of infection (Fig. 4A, B), however, a number of genes including Hexb, Glα, Ctsa, Ctsd, Ctsl, Man1a and Man2a1 were uncovered to be negatively controlled by IL-10 at late stage (Fig. 4C). To validate these data, we continuously evaluated the expression of proteins encoded by these genes by Western blot at 4h and 48h post-infection. In parallel with the observation from RT-PCR, the marked induction of Hexb and Ctd but not CtsZ proteins in IL-10-deficient cells was only observed at 48h post-infection whereas no difference was observed at earlier infection as compared with the control (Fig. 4D).

The observation that inhibition of IL-10 induced the phagolysosome fusion suggested that not only the expression but the delivery of lysosomal enzymes to Brucella phagosome might be also increased in the IL-10 lacking cells. For this, we monitored the fraction of Brucella phagosomes that could be labeled for CtsA or CtsD markers. At 48h after infection, the colocalization of Brucella phagosomes with both CtsA (Fig. 5A, B) and CtsD (Fig. 5C, D) was notably elevated in IL-10-deficient cells relative to controls, suggesting that IL-10 controls the recruitment of CtsA and CtsD by BCPs. However, these results also raised a question whether this effect is general to all of lysosomal enzymes including those that were not altered by IL-10. To figure out this question, the colocalization of CtsZ was observed by microscopy. However, the percentage of colocalization for these proteins was not influenced by IL-10 (Fig. 5E, F).

In order to clarify whether IL-10 could also have inhibitory effect on the expression of phagolysosome-related genes in normal condition, we treated RAW 264.7 cells with IL-10 siRNA and incubated for 2 days. The expression of 14 representatives was assessed by qRT-PCR, however, our data showed that no difference between IL-10 siRNA-treated cells and control was observed (Fig. S1A), suggesting that the inhibitory effect of IL-10 only occur in Brucella infected condition. Furthermore, the above data led to an argument that IL-10 might play a similar role in primary mouse macrophages. To address this assumption, we collected and differentiated the bone marrow-derived macrophages (BMM) from BALB/c mouse and treated with rIL-10 during Brucella infection. As expected, treatment with rIL-10 could induce the persistence of Brucella within BMM cells at late infection (Fig. S1B). In parallel, the down-regulation of trafficking regulators and lysosomal enzymes was
also obtained in rIL-10-treated cells at 4h pi compared with control (Fig. S1C, D). Moreover, the reduced colocalization of LAMP-1 with BCPs was observed when IL-10 signaling was enhanced (Fig. S1E), suggesting that IL-10 also suppresses phagolysosome fusion event aimed to promote bacterial survival in primary mouse macrophages. However, the target phagolysosomal genes of IL-10 signaling were different between BMM and RAW 264.7 cells that may result from the different regulatory mechanisms activated in responses to pathogens in these cells (15).

In summary, these results are the first evidences that clearly show the regulatory role of IL-10 on the lysosomal-mediated killing of \textit{B. abortus} in murine macrophages.

\textbf{Repressive effect of IL-10 on the phagolysosome fusion in \textit{Brucella}-infected macrophages is through Stat3-independent pathway}

After binding of IL-10 to extracellular domain of IL-10 receptor (IL-10R), various subsequent signaling pathways are activated; however, Jak1/Stat3 is a best understood pathway to be mainly responsible for subsequent transduction (16, 17). Thus, we hypothesized that the suppressive effect of IL-10 on phagolysosome event is through Jak1/Stat3 pathway. To test this hypothesis, we first used fluorescence microscopy to monitor the translocation of Stat3 to the nucleus upon treatment of \textit{B. abortus}-infected RAW 264.7 with either recombinant IL-10 (rIL-10) or IL-10 siRNA. Interestingly, the translocation of Stat3 into nucleus was remarkably enhanced when the infected cells were treated with rIL-10 whereas the reversion was observed with treatment of IL-10 siRNA (Fig. 6A, B). Likewise, evaluation of the activation of Stat3 by Western blot assay was also shown to be consistent with microscopy observation (Fig. 6C).

To determine the role of Jak1/Stat3 pathway in IL-10 signaling, the infected cells were concomitantly treated with rIL-10 and Stat3 siRNA, and the transcriptional level of IL-10-regulated trafficking regulators and hydrolytic enzymes were then assessed at 48h post-infection. Surprisingly, the transcriptional level of all IL-10-regulated trafficking regulators and hydrolytic enzymes were found to be unchanged when Stat3 pathway was blocked (Fig. 6D, E). In addition, the acquisition of LAMP-2 by BCP was not different between Stat3-blocking and –producing rIL-10-treated cells (Fig. 6F), suggesting that Jak1/Stat3 pathway is not responsible for the suppressive effect of IL-10 on phagolysosome fusion in \textit{B. abortus}-infected macrophages.

\textbf{IL-10 represses expression of pro-inflammatory cytokines by upregulating Socs3 expression during \textit{B. abortus} infection}

Although our data clearly indicated that the inhibitory role of IL-10 on lysosomal-mediated killing is independent with Jak1/Stat3 pathway, we still assessed Stat3 function in inflammatory response upon infection when IL-10 pathway was interfered. For this, we evaluated the expression of Socs3 and pro-inflammatory cytokines (Il6, Tnf, Mcpl, Il1a and Il1b) by RT-PCR and indirect ELISA in infected cells concomitantly treated with rIL-10 and Stat3 siRNA. Interestingly, the addition of rIL-10 caused a significant increase of Socs3 that came along with a decrease of Il6, Tnf and Il1a but not Mcpl and Il1b mRNA level at 48h pi. However, this consequence was blocked when the cells were concomitantly treated with Stat3 siRNA (Fig. 7A). In parallel, the presence of secreted cytokines in culture supernatant was also shown to be consistent with the observation by RT-PCR (Fig. 7B). These findings suggest that IL-10 mediated activation of Stat3/Socs3 that inhibits the inflammatory response in \textit{B. abortus}-infected macrophages.

Our findings showed two distinct regulatory mechanisms of IL-10 in \textit{Brucella}-infected macrophages that are through Stat3-dependent and independent pathways, leading to an argument on the actual role of Stat3 in antibrucella suppression by IL-10. For this, we concomitantly treated RAW 264.7 cells with rIL-10 and Stat3 siRNA, and then evaluated bacterial survival. Surprisingly, the treatment of Stat3 siRNA markedly reduced the rIL-10-promoted bacterial persistence in RAW 264.7 cells (Fig. 7C), suggesting that Stat3 is required for the antimicrobial suppression of IL-10 during \textit{B. abortus} infection in macrophage cells.

Taken together, our data clearly indicate that IL-10 regulates the proinflammatory antibrucella
immunity through controlling Stat3/Socs3 pathway in RAW 264.7 macrophages.

DISCUSSION

*Brucella abortus*, a causative agent of brucellosis, is one of the pathogens which have acquired the ability to survive and replicate within host cells by mechanisms that still needed to be elucidated. To date, several studies have shown that the fusion of bacteria-containing phagosomes with late endosomes/lysosomes is the most important innate immune effector against intracellular *Brucella*; however, the question as to how this process is operated by host and subverted by bacteria is not fully understood. Thus, identification of potential molecules which may control this process will provide insights into the rational therapeutic design for brucellosis elimination.

In agreement with previous observation, we also showed that *B. abortus* infection markedly induces expression of IL-10 (7, 9). In this study, we proved that this induction is beneficial for survival of intracellular *Brucella* since suppression of IL-10 signaling by siRNA treatment enhanced killing and restricted bacteria within macrophages. Consistent with a previous study (9), our study also showed that IL-10 promotes survival of intracellular *Brucella* by increasing bacterial ability to prevent the recruitment of lysosomes by BCPs, and this regulatory role of IL-10 was found at both early and late stage of infection.

It has been shown that IL-10 negatively regulates the expression of 3 regulators including Lamp1, Lamp2 and Rab34 at early phase whilst those at late stage were Lamp1, Lamp2, Rab5a, Rab7, Rab20, Rab22a, Rab34, and Stx11. The observed up-regulation at mRNA level resulted in a higher content of these proteins, suggesting that IL-10 mediates these proteins to control the fusion of phagosomes with late endosomes/lysosomes during *B. abortus* infection. These data could be rationalized by the findings that 8 potent membrane trafficking molecules are subverted by IL-10 during infection.

To date, LAMP-1 and Rab7 were the only regulators that have been proven to be crucial in regulating the fusion of BCPs with lysosomes (18) whereas the roles of other regulators are unknown. Our results showed that IL-10 negatively regulates Rab5A, Rab20 and Rab22A during infection, however these proteins have been found to be mainly associated to phagosomes containing intracellular pathogens such as *Listeria* and *Mycobacteria* and stimulate the maturation of these phagosomes at early infection (13), leading to the question whether these functional proteins also play undescribed roles in late *Brucella* infection. The other particularly interesting result is that LAMP-2, Rab34 and Stx11 which are also highly potential to regulate the fusion of phagosomes and lysosomes in macrophages. LAMP2 was demonstrated to play overlapped functions with LAMP-1 in recruiting Rab7, moving toward microtubule-organizing center and subsequently fuse with lysosomes (11). Likewise, Rab34 was also reported to be required for the fusion of phagosomes and lysosomes since deletion mutant of this gene markedly reduced the fusion ability (13, 19). Stx11 is another membrane-trafficking regulator that is suppressed by IL-10; however the general function of this protein in phagolysosome event is still unknown. Taken together, these findings suggest the potential of these trafficking regulators in governing the phagolysosome fusion since IL-10 subverts these proteins followed by inhibition of this effector.

Likewise, several hydrolytic enzymes including HexB, Gla, CtsA, CtsD, CtsL, Man1a and Man2a1 were clearly shown to increase when IL-10 signaling was inhibited at late stage of infection. Different members of cathepsins including CtsB, CtsD, CtsG, CtsL and CtsS are known to interact and contribute to killing intracellular *Mycobacterium tuberculosis* (20, 21), *Mycobacterium bovis* (22), *Streptococcus pneumoniae* (23) and *Listeria monocytogenes* (24). Additionally, HexB was also proven to protect macrophage against *Mycobacterium marinum* (25) in macrophages. Thus, the observation of induced fraction of phagosomes that are labeled by HexB and CtsD in BCPs paralleled with elevated fraction of phagosomes labeled with LAMP-1, suggesting that IL-10 might mediate these enzymes to inhibit lysosomal-mediated killing of *Brucella* within macrophages, and this also opens...
the argument on the roles of these lysosomal enzymes in killing *Brucella*.

On the other hand, our observation was found to be similar with previous reports on anti-inflammatory role of IL-10 when blocking of IL-10 up-regulated production of inflammatory cytokines during infection. However, to date, TNF is only one which was recently proven to participate in brucellacidal activity (26); the actual roles of another pro-inflammatory cytokines in IL-10 signaling as well as in brucellacidal immunity are yet to be investigated. Additionally, one of our striking findings in this study is that IL-10 inhibits phagolysosome fusion and pro-inflammatory brucellacidal activity through two distinct signaling mechanisms in which Stat3 importantly employs the pro-inflammatory brucellacidal suppression effect without phagolysosome interference. Thus, further investigations of Jak/Stat pathways might reveal the relation of inflammation and anti-*Brucella* activity which could be useful for further understanding of host resistance to *Brucella* infection.

In summary, our findings reveal a possible novel role of IL-10 to suppress the synthesis and delivery of molecules involved in phagolysosome fusion, which prevents killing of intracellular *B. abortus* in macrophages. In addition, further investigation of our identified molecules (8 trafficking regulators and 7 lysosomal enzymes which is regulated by IL-10) might provide insights into how this process is operated.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Mouse IL10, Stat3 siRNAs and rat anti-LAMP-1, -LAMP-2, mouse anti-Rab34, -Rab22a, -CtsZ, goat anti-CtsA antibodies were obtained from Santa Cruz Biotechnology, Texas, USA. Goat anti-CtsD antibody was obtained from R&D Systems, MN, USA. Texas red-rabbit anti-rabbit IgG antibodies and mouse IL-10 ELISA kit were purchased from Abcam, MA, USA. Mouse IL-10 recombinant protein, rabbit phosphor-Stat3 polyclonal, rabbit anti-HexB and FITC-goat anti-mouse IgG antibodies were obtained from Thermo Fisher Scientific, MA, USA while Texas red-goat anti-rat IgG antibody and Lipofectamine RNAiMAX were purchased from Life Technologies, CA, USA. FITC-conjugated goat anti-rabbit IgG antibody was obtained from Sigma-Aldrich Corp, Missouri, USA.

**Bacterial strain and cell culture**

*B. abortus* 544 biovar 1 strain was routinely cultured in Brucella broth (BD Biosciences, USA) at 37°C until stationary phase. The murine macrophage RAW 264.7 cells were grown at 37°C in 5% CO₂ atmosphere in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS) with or without 100 U/ml penicillin and 100 µg/ml streptomycin.

**Bone marrow-derived macrophage preparation**

Bone marrow-derived macrophages (BMM) from female BALB/c mice were prepared as previously described (27). Briefly, bone marrow cells were collected and cultured in BMM medium containing L-cell conditioned medium for 5 days at 37°C in 5% CO₂. The equal volume of fresh BMM medium without antibiotics was added and the cells were incubated further for 5 days. After 10 days of incubation, BMMs were washed three times with PBS and incubated with fresh RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) for further experiments.

**Bacterial infection and intracellular replication assay**

This assay was performed as previously described (3). Briefly, macrophages (10⁶ cells) were seeded in 96 well plate and incubated for 24h at 37°C in 5% CO₂. The cells were then infected with 10⁷ CFU of the virulent *B. abortus* for 1h at 37°C in 5% CO₂. The RPMI 1640 medium containing 10% (v/v) FBS and gentamicin (30µg/ml) were subsequently added to kill extracellular bacteria. At 2, 24 and 48h post-infection, cells were lysed and plated on Brucella agar plates for CFU determination. Additionally, the culture supernatant and the total of proteins or RNA from macrophages were also obtained at different time points.
RNA interference

RAW 264.7 cells were grown to 50% confluence on 6, 12 or 96 well plates and transfected siRNA directed against IL10 using Lipofectamine RNAiMAX. The cells were incubated for 24h at 37°C and 5% CO₂ prior to the performance of intracellular growth assay or protein, RNA isolation. The same concentration of negative control siRNAs were used throughout as controls. Knockdown efficiency was quantified using RT-PCR.

RNA extraction

The total RNA was isolated from RAW 264.7 cells (uninfected or infected with B. abortus) at different time points using a Qiagen RNeasy kit. DNA was removed before final elution of the RNA sample using the Qiagen “On-Column DNase Digestion” protocol.

RT-PCR

Real-time PCR analysis was performed as previously described (12). Briefly, the mixture of SYBR Green PCR master mix (Applied Biosystems) and different pairs of 10 pM primers (Table 1) were denatured at 95°C for 10 min and followed by 40 PCR cycles of 95°C for 15 s, 55°C for 30 s, and 60°C for 32 s. The mRNA expression profiles were normalized with respect to β-actin. Fold increase of each gene was calculated using the \(2^{-\Delta\Delta CT}\) method.

Western blot assays

The lysates of cells were identified by Western blot assay as previously described (4, 12). Briefly, the proteins were boiled for 5 min at 100°C in 2x SDS buffer and subjected to electrophoresis in 10% SDS PAGE gels. The separated proteins were then transferred onto Immobilon-P membranes (Milipore, MA, USA) using a semi-dry electroblot assembly (Bio-Rad, CA, USA). The membranes were blocked with 5% skim milk (Difco, MI, USA), and were subsequently incubated with primary antibodies (1:5,000 - 1:1,000 dilution) in blocking buffer. After wash with 0.05% PBS-T, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1:10,000 dilution; Sigma, Missouri, USA) in blocking buffer. The proteins were detected with ECL solution (Thermo Fisher Scientific, MA, USA).

LAMP-1, LAMP-2, Rab22A, CtsA, CtsD and CtsZ staining

The colocalization of Brucella-containing phagosomes (BCPs) with LAMP-1, LAMP-2, Rab22A, CtsA, CtsD and CtsZ was performed as previously reported (26, 28). Briefly, RAW 264.7 cells were treated with IL10 siRNA prior to infection. The infected cells were incubated 2h (LAMP-1) and 48h (LAMP-2, Rab22A, CtsA, CtsD and CtsZ), fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with blocking buffer (2% goat serum in PBS). The samples were stained with primary antibodies that were diluted 1:100 in blocking buffer that followed by secondary incubation with Texas red-goat anti-rat IgG or Texas red-rabbit anti-goat IgG (1:1000) in blocking buffer. The samples were stained with anti-B. abortus rabbit serum and FITC-conjugated anti-rabbit IgG to identify the bacteria and placed in mounting media. Fluorescence images were captured using a laser scanning confocal microscope (Olympus FV1000, Japan) and processed using FV10-ASW Viewer 3.1 software. 100 cells were randomly selected, and the colocalization percentage of these proteins with the BCPs was determined.

ELISA

The levels of IL-10, TNF, IL-6, IL-1β, IL-1α and MCP-1 in culture supernatants were determined by sandwich ELISA performed in accordance with the manufacturer’s instructions (Thermo Fisher Scientific, MA, USA).

Statistical analysis

The data are expressed as the mean ± standard deviation (SD). Student’s t-test was used to statistically compare the groups. Results with \(P < 0.05\) were considered significantly different.
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Conflict of interest

The authors declare that they have no conflict interest.

Author contributions

H. T. H., H. J. L., W. M., M. H. R., H. H. C. and S. K. designed the study. H. T. H. performed the experiments, analyzed the data and wrote the manuscript. A. W. B. R, H. T. X. N. and L. T. A. revised the manuscript. All the authors approved the final version of the manuscript.

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**Footnotes**

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**List of abbreviations**

TNF; tumor necrosis factor, IL; interleukin, MOI; multiplicity of infection, PBS; phosphate buffer saline, FBS; fetal bovine serum, CFU; colony forming unit.
### Table 1. List of primer sequences used for RT-PCR

| Gene     | Common name                          | Forward primer                  | Reverse primer                  |
|----------|--------------------------------------|---------------------------------|---------------------------------|
| b-actin  | β-actin                              | 5' - CGCCACCAGTTCGCCATGGA-3'    | 5' - TACAGCCAGGGAGCATCGT-3'     |
| Il1β     | Interleukin 1β                       | 5' - CAACCACACAAGTGATATTC-3'    | 5' - GGATCCACACTCTCCAGCTG-3'    |
| Il1α     | Interleukin 1α                       | 5' - CTTTCTATGATGCAAGCTA-3'     | 5' - GTTGCTGATACGTGACCC-3'      |
| Mcp1     | C-C motif chemokine 2                | 5' - GAGGTCTCTGTGCTGACCCCA-3'   | 5' - GCTTCAGAGTTACGGGTCAA-3'    |
| Il6      | Interleukin 6                        | 5' - TCCAGTGTGTCTTTGGGAC-3'     | 5' - GTACTCCAGAAAGACCAGAG-3'    |
| Tnf      | Tumor necrosis factor                | 5' - CACAGAAAGCAGATCCCGCG-3'    | 5' - CGGCAGAGAGGAGTGTGACT-3'    |
| Il10     | Interleukin 10                       | 5' - TGGCAGAAATCAAGGAGC-3'      | 5' - CAGCAGAATCAATACACACT-3'    |
| Socs3    | Suppressor Of Cytokine Signaling 3   | 5' - GACCAGCGCCACTCTTCACG-3'    | 5' - GTTCCGTGGGTGGCAGAGAA-3'    |
| Rab1     | Rab1                                 | 5' - CCTTCAATAACGGTAAACAGT-3'   | 5' - TAGTCTACTACTTTTTGTGG-3'    |
| Rab5a    | Rab5a                                | 5' - GTACTACCGAGGAGCAAG-3'      | 5' - AAGCTGTGTGTACATCGCAAG-3'   |
| Rab5b    | Rab5b                                | 5' - GACTAGCAGAAGTGACCCAG-3'    | 5' - CAATGCTGTTCCTGGTATT-3'     |
| Rab7     | Rab7                                 | 5' - CCTCTAGGAAAGAAGTGTTG-3'    | 5' - TTCTTGACCGGCTGTTGCCA-3'    |
| Rab9     | Rab9                                 | 5' - GCCCATGCAGATTGGGACAC-3'    | 5' - GCCGGCTTGGGCTTCTGTA-3'     |
| Rab10    | Rab10                                | 5' - GCCGAATGTTACTAGGGAACAG-3'  | 5' - GCCGCCTCTCCACACTGTGATA-3'  |
| Rab11    | Rab11                                | 5' - GAGCAGTAAGTGCCCTATTGG-3'   | 5' - GAACCTGCCCTGAGATGACGT-3'   |
| Gene    | Function                                      | 5' Sequence | 3' Sequence |
|---------|-----------------------------------------------|-------------|-------------|
| Rab14   |                                              | 5'-GCCGGAGCTACTATAGAGGAGCT-3' | 5'-GCCGTTCCTGATAGATTTCCTTG-3' |
| Rab20   |                                              | 5'-CTGCTGCAGCGCTACATGGAGCG-3' | 5'-CTCCGCAGCCTACAGGGAGC-3' |
| Rab22a  |                                              | 5'-GCCGACAAGAACGATTTCGTGCA-3' | 5'-GCCGACTTCTTGCGCTGGTCTC-3' |
| Rab24   |                                              | 5'-GCAGCGGGTGAGACCGCAGCCAG-3' | 5'-GCCTCAGACCCAACCCCAGC-3' |
| Rab31   |                                              | 5'-GCCCAATGAAGAGTCAGCAAGTC-3' | 5'-GCCACCTTGAGATGCTGGCGC-3' |
| Rab32   |                                              | 5'-GCCGAGTATATACATGAGAAGGCT-3' | 5'-GCCCTGGGAGACCTCTGGTG-3' |
| Rab34   |                                              | 5'-GCAGAGGTCGCGCTGGGCGG-3' | 5'-GGGCGTCCGAAGACCTCGG-3' |
| Eea1    | Early endosome antigen 1                      | 5'-GCCCAATGAAGAGTCAGCAAGTC-3' | 5'-GCCACCTTGAGATGCTGGGC-3' |
| Rilp    | Rab-interacting lysosomal protein             | 5'-CAGGAACAGCTACAGCGCCTCCT-3' | 5'-CTGAGGTTGCCGCATCGGTT-3' |
| Sort1   | Sortilin1                                     | 5'-GGGGAGCTGCAGCGCCTTTGC-3' | 5'-GGAGGCGCGCGGCGGCGGC-3' |
| Lamp1   | Lysosomal membrane glycoprotein 1            | 5'-GGCCGCTGCCTCTGCTGGCTG-3' | 5'-ATATCCTCTTCCAAGAATTG-3' |
| Lamp2   | Lysosomal membrane glycoprotein 2            | 5'-AGGGTACTTGCCCTTTATGCAAT-3' | 5'-GTGTCGCTTGGTCAGGACTG-3' |
| Stx2    | Syntaxin 2                                    | 5'-TGCCGTGCAGCGCCTGCCC-3' | 5'-GGTCCGCATCCCAACCCGC-3' |
| Gene   | Function                        | 5’-Sequence                                      | 3’-Sequence                                      |
|--------|---------------------------------|--------------------------------------------------|--------------------------------------------------|
| Stx3   | Syntaxin 3                       | 5’-GATGACACGGACGAGGTTGAGAT-3’                    | 5’-GTTGTGAGCTGTTCAAGGTCATC-3’                    |
| Stx4a  | Syntaxin 4A                      | 5’-CCCACGAGTGTGAGGCAGGGG-3’                      | 5’-GGCGTGGGCGAGGATGGTGACC-3’                     |
| Stx5a  | Syntaxin 5A                      | 5’-CGGGATCGGACCAGGACTTC-3’                       | 5’-CAAAGAGGGGACTTGGCCTTTT-3’                     |
| Stx6   | Syntaxin 6                       | 5’-TCAACACTGCCAAGGATTGT-3’                       | 5’-GTTCATCGAGGTGCTCCAGATCC-3’                    |
| Stx7   | Syntaxin 7                       | 5’-GGAAGCGGCCGAGTCAGGGTG-3’                      | 5’-CATTTGTGTGATCTTTTGGATGTTAG-3’                 |
| Stx8   | Syntaxin 8                       | 5’-GGCAGGACTGCACCATGGCC-3’                       | 5’-GTCTTCGATCGCCCTCCAGTTGTG-3’                   |
| Stx11  | Syntaxin 11                      | 5’-GCTTCAAGAATTGTCCAGGAT-3’                      | 5’-ATGGACGTGAGGAAGCCGAGT-3’                      |
| Stx12  | Syntaxin 12                      | 5’-CCGGTCTCTGCTCAGTGTCATGTC-3’                   | 5’-GTGGGCTTGGCTGATCCGGTGGATG-3’                   |
| Stxbp1 | Syntaxin-binding protein 1       | 5’-CGGAGCCGAAGACTCGAAGAACG-3’                    | 5’-CAGCAGGAGGACAGCATCCTCATG-3’                   |
| Stxbp2 | Syntaxin-binding protein 2       | 5’-CTTCAGGGGAGATGCGGCCC-3’                       | 5’-CAACAGGATGACAAGATTCGCATG-3’                   |
| Lyz1   | Lysozyme 1                       | 5’-CTCTCTGACTGTCGAGTGG-3’                        | 5’-CTGAGCTAAACACACCAGTCAGC-3’                    |
| Lyz2   | Lysozyme 2                       | 5’-GGCCAAGGTCTCAATCGTTGTG-3’                     | 5’-GCAGAGCACTGCAATTGATCCCA-3’                    |
HexA  Hexosaminidase A  5'-GCCGGCTGCAGGCTCTGGGTTTC-3'  5'-GCGCGGCGAAGCTGACATGGTAC-3'

HexB  Hexosaminidase B  5'-CCCAGGCTGCTGCTGCTGCTGCAGGC-3'  5'-GTGGAATTGGCTGCTGTCGATG-3'

Hexdc Hexosaminidase D  5'-CCACGCCATTTAAGTAACTGACATGGTAC-3'  5'-GGCCCTCAGCAGCTCAGGTGGCC-3'

Gla  Galactosidase, α  5'-GGCCATGAAGCTTTTGAGCAGAG-3'  5'-AGTCAAGGTCACATGAAACGTT-3'

Glb1  Galactosidase, β1  5'-GGAGGTGCAGCGGCTGGCCAGAGC-3'  5'-GTGCATCGTGACGCTCAGACGAAG-3'

Glb1l Galactosidase, β1 like  5'-GCCCTCCCCGCTGCTGGCGAAGCAG-3'  5'-CTGTCATGTTCCGATCCAAAGC-3'

Lpl  Lipoprotein lipase  5'-GGAGGTGCAGCGGCTGGCCAGAGC-3'  5'-GGCCCTCAGCAGCTCAGGTGGCC-3'

CtsA  Cathepsin A  5'-GCCCTCCCCGGCTGCTGGCGAAGCAG-3'  5'-GCGCGGCTGCAAGGTGAAGG-3'

CtsB  Cathepsin B  5'-GCCCTCCCCGGCTGCTGGCGAAGCAG-3'  5'-GCACCGATCGCTCGATCCCTTC-3'

CtsC  Cathepsin C  5'-GCCGCCACACAGCTATCAGTTACTG-3'  5'-GCCCTCGAGAGCTCAGGTATGTCG-3'

CtsD  Cathepsin D  5'-GCGCCACACAGCTATCAGTTACTG-3'  5'-CACCTCGAGAGCTCAGGTATGTCG-3'

CtsE  Cathepsin E  5'-GCCGCCACACAGCTATCAGTTACTG-3'  5'-GCGCCACACAGCTATCAGTTACTG-3'
| Protein | Description | 5'-Sequence | 3'-Sequence |
|---------|-------------|-------------|-------------|
| CtsF    | Cathepsin F | 5'-GCC GCA GGC TCC GCC TCG-3' | 5'-GCC GCT CCT AGC ACG GCC-3' |
| CtsG    | Cathepsin G | 5'- CCTGTGCACACCTGTATCATAAA-3' | |
| CtsH    | Cathepsin H | 5'- CTGAGAACCCTTCTCTTCCAAGAGC-3' | 5'- AGCAGCCAGCCACCCAGCAGCAGC-3' |
| CtsK    | Cathepsin K | 5'- GGATGAAATCTCTCGGCGTTTAA-3' | 5'- GTCTCCCAAAGTGGTTCATGGCCAG-3' |
| CtsL    | Cathepsin L | 5'- GCCGGCTTCTCTGCTGGTGC-3' | 5'- GCCCTCCATTGAAAGCCGTGC-3' |
| CtsO    | Cathepsin O | 5'- GCCGACTACCATTGGGATCTCTGGA-3' | 5'- GCCGTCTCCATGGGGGTA TCTGGG-3' |
| CtsS    | Cathepsin S | 5'- GCCGTCTCCATTGAAAGCCGTGC-3' | 5'- GCCGTCCTTCTGCTGGGTA TCTGGG-3' |
| CtsZ    | Cathepsin Z | 5'- GCCGTCGTCGGGGTCGGTGCAGCA-3' | 5'- GCCGTCTCCATTGAAAGCCGTGC-3' |
| Man1a   | Mannosidase 1, α | 5'- CAAGCTGCTCAGCGGGGTTCTGGT-3' | 5'- GCCGATCTGGCTAAGTCTC-3' |
| Man1a2  | Mannosidase 1, α2 | 5'- GAAAAGCTTCCGGAGTCAATTCAAG-3' | 5'- CTTCCCAGCCCCACTGGCTGTTATC-3' |
| Man2a1  | Mannosidase 2, α1 | 5'- GCTACA GACATTTTGT GCCATATG-3' | 5'- CTGGGGGAACTCCCCAGGGACAC-3' |
| Man2a2  | Mannosidase 2, α2 | 5'- GGATAGAACAGCTGGAACACAA CTGC-3' | 5'- CCCGTCCTCCCCCAAAGCAAACTGG-3' |
| Protein   | Description                  | 5' - Primer Sequence                      | 3' - Primer Sequence                      |
|-----------|------------------------------|------------------------------------------|------------------------------------------|
| Man2b1    | Mannosidase 2, α B1          | 5' - G TGATGTTCAG CACGCATCTG TTC-3'      | 5' - CGTACAGCGTCCTGGTTGCACTG-3'           |
| Man2b2    | Mannosidase 2, α B2          | 5' - CCGTCTTCCC AGAGCCACCC CCAG-3'       | 5' - CAGAGGACGTGGGCCTCCGAAC-3'            |
| Man1c1    | Mannosidase α Class 1C Member1 | 5' - GAGGCCATAG AGACCTATCT CGTG-3'      | 5' - CATGGCAGCTGCTGATCTGGG-3'            |
| Man2c1    | Mannosidase α Class 1C Member1 | 5' - GTAGCCTGCA ATGGGCTTCT GGGG-3'      | 5' - CAACAGCTCCAGGTCCACCAGGAG-3'         |
FIGURE LEGENDS

Figure 1. IL-10 promotes *B. abortus* survival by suppressing phagolysosome fusion. Macrophages infected with *B. abortus* and the transcriptional and translational profiling of II10 was examined by qRT-PCR (A) and sandwich ELISA (B). Bacterial intracellular growth was evaluated in cells with or without treatment of IL10 siRNA (C). The colocalization of BCPs with LAMP-1 was analyzed at 48h post-infection. The LAMP-1 positive (arrows) or negative bacteria (arrow heads) was visualized by fluorescence microscopy (D) and the percentage of LAMP-1 colocalized with BCPs in 100 cells were determined (E). Data represent the mean ± SD of triplicate experiments. Asterisk indicated the significant difference ($P<0.05$), Scale bars = 5μm.

Figure 2. IL-10 significantly regulates the expression of membrane-trafficking regulators. Macrophages were treated with IL10 siRNA prior to *B. abortus* infection. Total RNA content was isolated and the expressions of representative membrane-trafficking proteins were evaluated by RT-PCR at 4h (A), 24h (B) and 48h (C) post-infection. The expression of LAMP-2, Rab22A and Rab34 proteins was checked at 4 and 48h post-infection by Western blot (D). Data represent the mean ± SD of triplicate experiments. Asterisk indicated the significant difference ($P<0.05$).

Figure 3. IL-10 inhibits the acquisition of trafficking regulators in *B. abortus*-infected macrophages. Macrophages were treated with IL10 siRNA prior to *B. abortus* infection. The fraction of BCPs that could be labeled for LAMP-2 (A) and Rab22A (C) were evaluated by immunofluorescence assay. Arrow or arrowhead indicates marker positive or negative bacteria, respectively. At least 100 cells were counted to determine the percentage of colocalization of LAMP-2 (B) and Rab22A (D). Data represent the mean ± SD of triplicate experiments. Asterisk indicated the significant difference ($P<0.05$).

Figure 4. IL-10 contributes to controlling the synthesis of lysosomal hydrolases. Macrophages were treated with IL10 siRNA and then infected with *B. abortus*. Total RNA was isolated at indicated times. The expression of representative lysosomal hydrolases was evaluated by RT-PCR at 4h (A), 24h (B) and 48h (C) post-infection. The total cellular content of CtsD, CtsZ and HexB proteins was checked at 4 and 48h post-infection by Western blot (D). Data represent the mean ± SD of triplicate experiments. Asterisk indicated the significant difference ($P<0.05$).

Figure 5. IL-10 suppresses normal phagolysosome fusion during *B. abortus* infection. Macrophages were treated with IL10 siRNA prior to *B. abortus* infection. The fraction of BCPs that could be labeled for CtsA (A), CtsD (C) and CtsZ (E) were evaluated by immunofluorescence assay. At least 100 cells were counted to determine the percentage of colocalization of CtsA (B), CtsD (D) and CtsZ (F). Data represent the mean ± SD of triplicate experiments. Asterisk indicated the significant difference ($P<0.05$), Scale bars = 5μm.

Figure 6. IL-10 suppresses phagolysosome fusion by Stat3-independent pathway during *B. abortus* infection. Infected RAW 264.7 cells were treated with either recombinant IL-10 (rIL-10) or IL10 siRNA and subjected to fluorescence microscopy observation of Stat3 translocation (A). The quantitative kinetic analysis of Stat3 activation was determined in at least 100 cells in each experiment (B). The activation of Stat3 protein was evaluated by Western blot assay using anti-phospho-Stat3 antibody (C). To check the role of Stat3 in IL-10 signaling, RAW 264.7 cells were concomitantly treated with rIL-10 and Stat3 siRNA. Total RNA was then extracted and quantification of representative trafficking regulators (D) and hydrolytic enzymes (E) were measured by qRT-PCR. The fraction of BCPs that could be labeled for LAMP-2 was evaluated by immunofluorescence assay (F). At least 100 cells were counted to determine the percentage of colocalization of LAMP-2 (G). Data represent the mean ± SD of triplicate experiments. Asterisk indicated the significant difference ($P<0.05$).

Figure 7. Stat3/Socs3 plays a major role in anti-inflammatory effect of IL-10. The infected RAW 264.7 cells were concomitantly treated with rIL-10 and Stat3 siRNA. The transcriptional and translational profiles of Socs3 and pro-inflammatory cytokines were determined at 48h pi by RT-PCR (A) and sandwich ELISA
(B), respectively. The RAW 264.7 cells were concomitantly treated with rIL-10 and Stat3 siRNA, and the bacterial survival was evaluated (C). Data represent the mean ± SD of triplicate experiments. Asterisk indicated the significant difference ($P<0.05$).
Fig. 1

A) Graph showing fold change of IL-10 mRNA levels in Uninfected and Infected conditions over time.

B) Bar graph showing IL-10 concentration (log10 ng/ml) in Uninfected and Infected conditions.

C) Line graph showing log10 IFN-γ secretion in Control and IL10 siRNA conditions over time.

D) Fluorescence images of LAMP-1 expression in Control, IL10 siRNA, and merge conditions.

E) Bar graph showing percentage of LAMP-1 positive cells in Control, IL10 siRNA, and infected conditions.

Fig. 2

A) Heatmap showing fold changes of mRNA levels for various genes in different conditions.

B) Heatmap showing fold changes of mRNA levels for different genes in Control and IL10 siRNA conditions.

C) Heatmap showing fold changes of mRNA levels for various genes in different conditions.

D) Western blot images showing protein expression for E10 and Rabbit LAMP-2 under different conditions.
Fig. 3

A

LAMP-2

*B. abortus*

Merge

Control

IL10 siRNA

B

LAMP-2 colocalization (%)

PBS

IL10 siRNA

C

Rab22A

*B. abortus*

Merge

Control

IL10 siRNA

D

Rab22A colocalization (%)

PBS

IL10 siRNA

Fig. 4

A

B

C

D

Time (%)

PBS

*B. abortus*

CtsD

Hsc8

CtsZ

B-acGn

0

0.5

1

1.5

2

0.3

0.6

0.9

1.2

1.5

2

0

0.5

1

1.5

2

0

0.5

1

1.5

2
Fig. 7

A

B

C

Fig. 7
Interleukin 10 suppresses lysosome-mediated killing of Brucella abortus in cultured macrophages

Huynh Tan Hop, Alisha Wehdnesday Bernardo Reyes, Tran Xuan Ngoc Huy, Lauren Togonon Arayan, WonGi Min, Hu Jang Lee, Man Hee Rhee, Hong Hee Chang and Suk Kim

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