MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2017; 23: 5420-5429
DOI: 10.12659/MSM.904681

Received: 2017.04.04
Accepted: 2017.05.24
Published: 2017.11.14

HLA-B27 Correlates with the Intracellular Elimination, Replication, and Trafficking of *Salmonella Enteritidis* Collected from Reactive Arthritis Patients

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Source of support: This work was supported by International Collaborative Grants of Sichuan Science and Technology Project (No. 2010HH0033

Background: The aim of this study was to explore the correlation between HLA-B27 and the intracellular elimination, replication, and trafficking of *Salmonella enteritidis* (*S. enteritidis*) collected from patients with reactive arthritis.

Material/Methods: Confocal microscopy, flow cytometry, and sandwich enzyme-linked immunosorbent assay (ELISA) were employed in this study to evaluate the localization of proteins of interest, to assess the intracellular trafficking of *S. enteritidis*, and to measure the production of cytokines of interest.

Results: HLA-B27 was negatively associated with intracellular *S. enteritidis* elimination in healthy human monocytes/macrophages. In *S. enteritidis* infected monocytes/macrophages, HLA-27B was also negatively correlated with bacteria elimination but positively related to bacteria replication. *S. enteritidis* did not co-localize with NRAMP1 and LAMP1/2 in HLA-B27 cells. *S. enteritidis* did not co-exist with transferrin or dextran within HLA-B27 and A2 cells.

Conclusions: HLA-B27 is closely associated with the intracellular elimination and replication of *S. enteritidis*. Replicated bacteria in HLA-B27 monocytic cells were located within unique vacuoles rather than disturbing host endocytosis.

MeSH Keywords: Arthritis, Reactive • Cytokines • HLA-B27 Antigen • Monocytes

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Background

Reactive arthritis (ReA) is caused by infections of the gastrointestinal or genitourinary tract [1]. The causative bacteria are usually *Salmonella, Yersinia, Shigella, Campylobacter*, and *Chlamydia*; and they usually cause infections in mucosal areas [1,2]. Up to 80% of patients with ReA have been reported to be HLA-B27 positive [3,4]. Although the strong association between HLA-B27 and spondyloarthropathies (SpAs) has been known for more than two decades, how HLA-B27 affects SpAs pathogenesis has not been thoroughly elaborated. In addition, HLA-B27 and *Salmonella enteritidis* (*S. enteritidis*) both have close relationships with ReA. Whether HLA-B27 is associated with the intracellular elimination, replication, and trafficking of *S. enteritidis* in ReA is of interest.

ReA-triggering bacteria share some characteristics; for example, they originally affect mucosal tissue areas and they have lipopolysaccharide (LPS) as a part of their outer membranes. Prolonged immune responses to ReA-triggering microbes have been found in ReA patients [1,5]. Serum antibody responses, especially IgA and IgG response, are usually strong and persistent in ReA patients compared with non-arthritis patients with the same infections [6]. A higher proportion of IgA-*Yersinia* antibodies in arthritis patients are of the secretory IgA2 form [7], which indicates a strong and continuous antigenic stimulation in the intestinal mucosa. Components of the causative bacteria have been shown to be sustained in the peripheral blood cells of ReA patients after *Salmonella* or *Yersinia* infection. It has been suggested that living bacteria in hosts continuously produce antigenic materials into the circulation and stimulate the immune reactions observed in ReA patients [1,8]. Elimination of *S. enteritidis* and *Yersinia enterocolitica* O: 3 and O: 8 is impaired in HLA-B27 positive cells *in vitro* [9,10], as well as in monocytes/macrophages from HLA-B27 positive persons with ReA history [11,12]. The mechanism behind this phenomenon remains obscure.

Growing evidence has shown that several bacteria and parasites (such as *Salmonella, Mycobacterium, Chlamydia trachomatis*, and *Leishmania*) evade host defenses by developing various strategies, even by replicating within macrophage phagosomes [13,14]. The phagosomes usually undergo a series of biochemical modifications via the host cell endocytic pathways. A plethora of biomarkers for the endocytic pathways have been used to recognize the changes in this process [15]. Rathman et al. reported that *S. typhimurium* lived in phagosomes that were not involved in the degradative pathway of the cells [16]. Biomarkers such as lysosomal glycoproteins (LAMP1 and LAMP2) and lysosomal acid phosphatase (LAP) are delivered to vacuoles through a mannose 6-phosphate receptor (M6PR)-independent mechanism [17,18]. To the contrary, phagosomes containing heat-killed organisms appear to be processed along the degradative pathway in monocytic cells [16,19,20]. The examination of the intracellular trafficking pattern of *Salmonella*-containing phagosomes would contribute to our understanding of how and why microbes survive in seemingly hostile host environment and whether HLA-B27 could affect the intracellular bacteria elimination by modulating their intracellular trafficking pathways.

We aimed to explore the correlation of HLA-B27 and intracellular elimination, *S. enteritidis* replication, and to investigate the intracellular trafficking pathways of *S. enteritidis* in HLA-B27 transfected monocytic cells. Immunofluorescence, flow cytometry, and sandwich enzyme-linked immunosorbent assay (ELISA) were primarily used in this study. We confirmed the association between HLA-B27 and intracellular elimination, trafficking and replicating of *S. enteritidis* within human monocytes/macrophages.

Material and Methods

Blood samples and human monocytes

Peripheral blood (120 mL) was drawn from 19 healthy donors (16 males and three females, average age 38.26±10.96 years). All of the donors did not take medications when the blood was drawn. Among the 13 HLA-B27 positive donors, six had ReA histories but without clinical manifestations at the time of the blood draw (HLA-B27$^+$/ReA$^+$), whereas the other seven donors had no ReA history (HLA-B27$^+$/ReA$^-$). Mononuclear cells were isolated using Ficoll-Pague reagents (Research grade, Amersham Pharmacia Biotech AB, Uppsala, Sweden) and monocytes were cultured as previous described [21]. The membrane HLA expression was detected using immunofluorescence. The monocytes were matured to be monocyte-derived macrophages with recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) as previous reported [22]. Maturation of the macrophages was verified by the change of morphology and expression of certain markers on the cell membrane.

Human monocytic cell line U937 transfection

The human monocytic cell line U937 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Full-length 6 kb HLA-B*2705 genomic DNA (B27g) or 5.1 kb HLA-A2 genomic DNA in pUC9 vectors were transfected by electroporation following published protocols [23,24]. Briefly, approximately 5×10^6 cells were cultured in RPMI 1640 with 10% fetal calf serum (FCS), 3% L-glutamine, and 50 µg/mL of gentamicin (Biological Industries, Beit HaEmek, Israel) at 37°C with 5% CO_2. Co-transfection of the HLA constructs and pSV2neo vectors were performed using a single pulse from a Gene Pulser (0.25 kV, 960 µF, Bio-Rad, Hercules, CA, USA).

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U937 cells in the mock group were transfected with pSV2neo plasmids only.

S. enteritidis infection

The green fluorescence protein (GFP) pACYC vectors (kindly provided by Dr. Denise Monack, Stanford University, CA, USA) were used to transform *S. enteritidis* isolated from a ReA patient using the same electroporation method described earlier. *S. enteritidis* was cultured in Luria-Bertani (LB) media until the optical density reached 0.5. Bacteria were put on ice, washed three times with 10% ice-cold glycerol, and suspended in 10% ice-cold glycerol (1: 200) for further experiments.

GFP-transformed *S. enteritidis* were grown at 37°C for 18 hours in LB media containing 29 μg/mL chloramphenicol. The chloramphenicol-resistant clone was selected and 500 μL of the *S. enteritidis* culture was cultured in 10 mL LB media containing 20 μg/mL chloramphenicol for five hours to reach the logarithmic growth.

The U937 cells were allowed to mature into macrophage-like cells by incubating with 10 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA) in tissue culture flasks (5×10^5 cells/well, 10 cm, Greiner, Frickenhausen, Germany) for 24 hours. Two hours before the infection, the media were replaced with RPMI 1640 containing 10% AB serum. The cells were thereafter incubated with *S. enteritidis* for one hour (multiplicity of infection (MOI) between 30: 1 and 50: 1). Before the media were replaced with RPMI 1640 containing 10% AB serum, 3% L-glutamine, and 50 μg/mL of gentamicin (Biological Industries, Beit HaEmek, Israel), the cells were washed three times with Hanks' balanced salt solution (HBSS).

Confocal microscopy

Confocal microscopy was used to evaluate the co-localization of GFP-*S. enteritidis* and membrane/lysosomal marker proteins including CD14, HLA-B27, LAMP1/2, and NRAMP1. The quantity of the intracellular *S. enteritidis* was calculated accordingly.

Briefly, 10 mm glass coverslips (Bellco Glass Inc., Vineland, NJ, USA) were placed on the bottom of 24-well plates (Greiner Labortechnik, Frickenhausen, Germany), approximately 5×10^5 HLA-B27 or HLA-A2 transfected U937 cells were added to each well and stimulated with PMA for 24 hours. At each indicating time point after infection, coverslips were washed twice, fixed with 3.7% formaldehyde for 10 minutes, permeabilized with 0.1% saponin for 10 minutes, and blocked with 2% bovine serum albumin for 10 minutes.

The primary antibodies used in the immunofluorescence include anti-human CD14 monoclonal antibodies (mAb), anti-human HLA-A2 mAb, anti-human HLA-B27 mAb (ME-1, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), anti-human LAMP1/2 antibodies (LAMP1 and LAMP2, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), and polyclonal rabbit anti-human NRAMP1 antibodies (from Dr. Kishi, Center for Gene Research, Yamaguchi University, Japan). The secondary antibodies used in the immunofluorescence include Alexa-conjugated goat anti-mouse antibody (for CD14 and ME-1), tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (H+L) (for HLA-A2), Alexa-conjugated goat anti-rabbit antibody (for NRAMP1, Alexa Fluor 568 TM goat anti-mouse IgG (H+L), F(ab')2 and goat-anti-rabbit IgG (H+L) F(ab')2, Molecular Probes, Leiden, Netherlands). Lysine fixable tetramethyl rhodamine dextran (fluoro-ruby, 10,000 MW and Texas Red dextran, 70,000 MW) and tetramethyl rhodamine conjugated transferrin were purchased from Molecular Probes (Molecular Probers Europe BV, Leiden, The Netherlands).

Cells were then visualized using a Leica TCS SP laser confocal microscope (488 nm excitation light, Zeiss, Jena, Germany). Rhodamine red fluorescence was collected using 568 nm excitation light using TCSNT package (0.5 nm/section). Leica confocal assistant program was used to format the merge the green and red images (Leica, Wetzlar, Germany). The percentage of bacteria-containing phagosomes that co-localized with the given marker was calculated by analyzing ≥50 phagosomes from random fields at each indicating time point.

The endocytic trafficking of *Salmonella*-containing phagosomes

The interaction of *Salmonella*-containing phagosomes with endocytic traffic was studied using fluoresceinated dextran and transferrin in PBS as previously described [16]. In preparation for confocal fluorescence microscopy, 200 μL rhodamine-conjugated transferrin in PBS (12.5 μg/mL) was incubated with cells after infection for 0.5 hours at 37°C. Then 200 μL Texas Red dextran (MW 70,000) or tetramethylrhodamine (10,000) (10 μg/mL) was incubated with cells for 0.5 hours at 37°C in preparation for confocal microscopy.

Colony forming unit (CFU) of intracellular bacteria

Cells were collected at each indicating time points (1 hour, 4 hours, 24 hours, 72 hours, and 7 days for monocytes, and 1 hour, 4 hours, 8 hours, 24 hours, and 72 hours for macrophages) to determine the quantity of intracellular bacteria. Briefly, 500 μL 1% Triton-X 100 (Sigma-Aldrich, St. Louis, MO, USA) was used to lyse cells for five minutes and pipetted vigorously to release intracellular bacteria. Thereafter, 20 μL was used for tenfold dilution in 1 mL PBS. Aliquots of 200 μL were added to 96-well microtiter plates and 20 μL of those dilutions to LB plates with 20 μg/mL chloramphenicol. Coverslips were

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prepared as mentioned above, and intracellular bacteria were measured using an incident light fluorescent microscope (Leitz, Wetzlar, Germany). Altogether, 100 cells were calculated from each coverslip. A cell with at least one GFP-bacterium inside was counted as a bacteria-containing cell.

Flow cytometry of intracellular bacteria measurement

To determine intracellular GFP-bacteria and their replication, cells were scraped off the bottles using rubber policemen at different time points after infection. The cells were collected and centrifuged at 4°C (1,000 rpm, five minutes). 5×10^5 cells/tube were added to the FACs tubes, washed once with Solution I (0.01% NaN_3 and 2% FCS in PBS) and centrifuged with Immufuge for 1.5 minutes at high speed. The supernatant was removed and the pellet was resuspended; 400 µL fix solution (1% formaldehyde in PBS) was added to each tube (4°C) for flow cytometry. Uninfected cells were the negative controls. The cells containing GFP-*Salmonella* were separated according to the fluorescence intensity at FL1. The cells with FITC intensity over 10^2 were considered to be high fluorescence intensity (HFI) cells, and were regarded as the cells with high bacteria replication.

ELISA

At each indicating time point, media were collected for cytokine measurement. The cytokines TNF-α and IL-10 were detected in cell-free supernatants using ELISA. The antibodies used in ELISA were anti-TNF-α (Mab1, Mab11) and anti-IL-10 (JES3-9D7, JES3-12G8) (Pharmingen, San Diego, CA, USA). The optical absorbance was measured at 405 nm using the VictorTM Multilabel Counter (Wallac Oy, Turku, Finland).

Statistical analysis

Statistical comparison of CFU and cytokine concentrations between HLA-B27 positive and negative cells was performed using paired two-tailed Student's *t*-test and one-way anova plus post hoc analyses. The Spearman's Rho test was used to calculate correlations.

Results

Elimination of intracellular *S. enteritidis* by monocytes/macrophages

Monocytes and macrophages isolated from the donors were challenged with GFP-labelled *S. enteritidis*. One hour after the infection was considered as the start of bacteria uptake. More bacteria were seen in HLA-B27$^+$/ReA$^+$ monocytes compared to HLA-B27$^-$/ReA$^-$ monocytes. Compared to the HLA-B27$^-$/ReA$^-$ monocytes, less intracellular bacteria were eliminated by HLA-B27$^+$/ReA$^+$ monocytes ($p<0.05$ at 4 hours, 24 hours, and 72 hours). Survival of intracellular bacteria in HLA-B27$^+$/ReA$^-$ monocytes tended to be better than that in HLA-B27$^-$/ReA$^-$ monocytes (Figure 1A).

After infection with the same bacteria, HLA-B27$^+$/ReA$^+$ macrophages took in a little bit more bacteria than HLA-B27$^-$/ReA$^-$ macrophages did with insignificant differences, and showed a different pattern of intracellular bacteria elimination (Figure 1B). The HLA-B27$^+$ macrophages were significantly less efficient in the elimination of intracellular bacteria than HLA-B27$^-$ controls at 24 hours and 72 hours post-infection ($p<0.05$, $p=0.05$, respectively).

In fluorescence microscopy, we counted at least 100–200 cells at 40x magnification within five random fields. The percentage of GFP-*S. enteritidis* containing cells was much greater in HLA-B27 cells (cells that are positive of HLA-B27 expression) than in HLA-A2 cells at 24 hours, 72 hours, and 7 days post-infection (Figure 1C, 1D). The two methods yielded slight differences in intracellular bacteria survival at different times; the relative inhibition on the bacteria replication in HLA-A2 cells was clearly defined.

If we set the ratio of living bacteria/cell at 30 minutes post-infection as a baseline, the increase of this ratio in HLA-B27 cells was substantially higher than in the control cells (Figure 1E). The unique intracellular replication pattern seen within HLA-B27 transfected cells might result in prolonged intracellular survival of *S. enteritidis* in these cells.

To detect the intracellular proliferation of *S. enteritidis*, we identified the percentage of GFP-bacteria positive cells with HFI using flow cytometry method. An obvious increased replication of GFP-bacteria in HLA-B27 cells was identified at 6 hours and 24 hours post-infection. About 1% of HLA-B27 transfected cells compared to only 0.1% HLA-A2 transfected cells were found to be the HFI cells (Figure 1F). The mean value of the gated HFI cells transfected with HLA-B27 was also greater than HLA-A2 cells at 6 hours, 24 hours, and 72 hours, indicating a larger number of GFP-bacteria inside the gated HLA-B27 cells than inside the gated HLA-A2 cells (Figure 1G).

This unique replication pattern of *S. enteritidis* in HLA-B27 cells was also seen by fluorescence microscopy. A larger number of replicated GFP-*S. enteritidis* (more than 100 bacteria within one single vacuole) were seen in HLA-B27 cells compared to HLA-A2 cells at 6 hours post-infection (Figure 1H).

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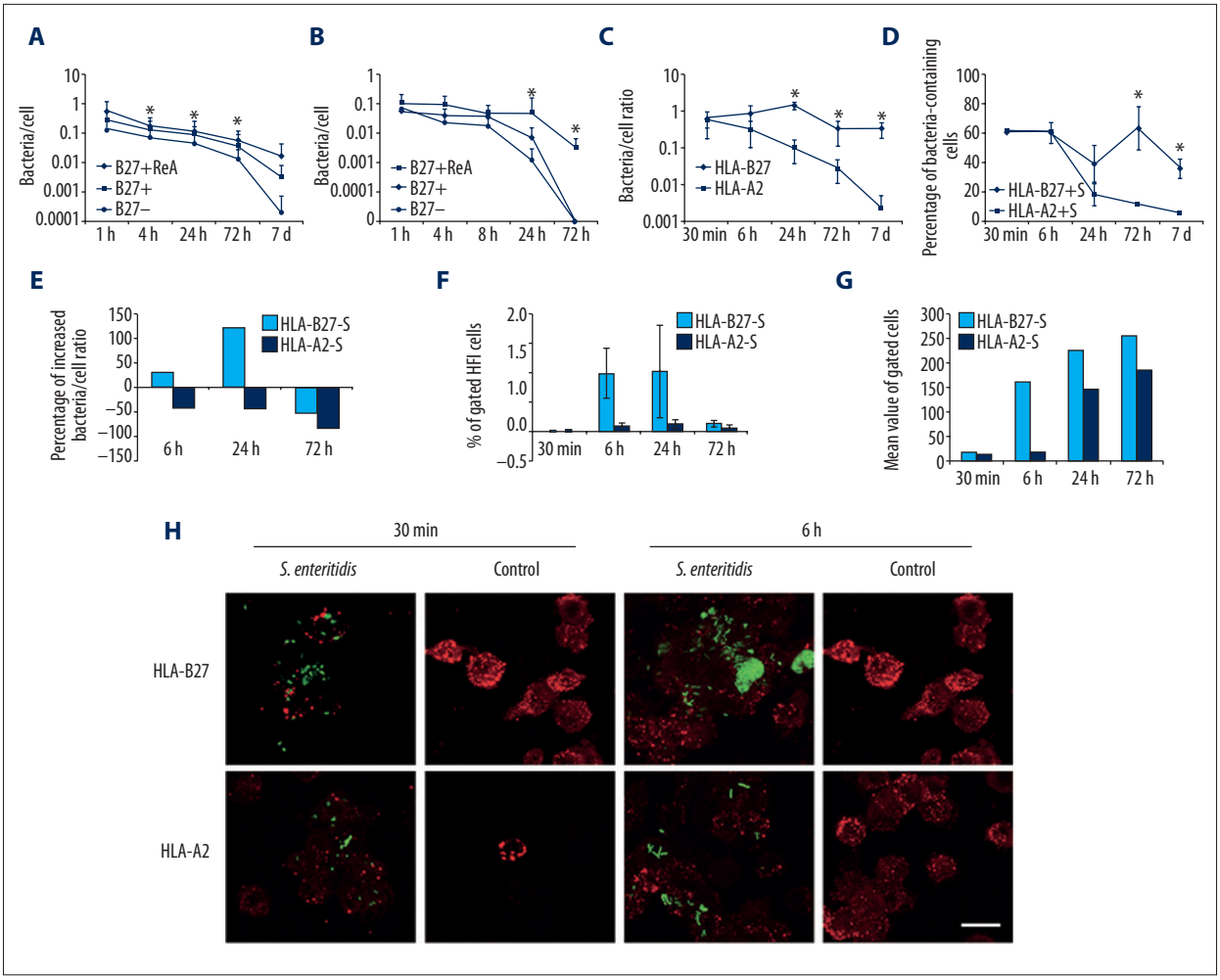

Figure 1. *S. enteritidis* survival within monocytes/macrophages. The uptake and intracellular survival of *S. enteritidis* in (**A**) monocytes and (**B**) macrophages. Impaired elimination of intracellular *S. enteritidis* was seen in HLA-B27 monocytes and macrophages. (**C**) Flow cytometry results illustrating the number of bacteria-containing cells in HLA-B27 and HLA-A2 groups. (**D**) Flow cytometry results illustrating the percentage of bacteria-containing cells in HLA-B27 and HLA-A2 groups. (**E**) The percentage of increased colony forming unit (CFU). HLA-B27-*S. enteritidis* showed significantly higher bacteria/cell ratio than HLA-A2-*S. enteritidis* cells. (**F**) The percentage of gated FITC positive cells with high fluorescence intensity (HFI). FITC intensity over 10^2 was considered with HFI, and HFI cells were regarded as the cells with large numbers of bacteria. (**G**) Mean value of gated bacteria-containing cells. HLA-B27-*S. enteritidis* showed significantly g mean value at any indicating time point than HLA-A2-*S. enteritidis* cells. (**H**) "Highly-replicated" bacteria in HLA-B27 cells. A larger number of replicated GFP-*S. enteritidis* (more than 100 bacteria within one single vacuole) were seen in HLA-B27 cells than in HLA-A2 cells at 6 hours post-infection. Scale bar: 10 µm. Green: GFP-*S. enteritidis*, Red: HLA proteins. Data were presented as mean value ±SEM. Experiments were done in triplicate and every measurement was done five times, * $p<0.05$.

Expression of TNF-α and IL-10 in monocytes/macrophages after *S. enteritidis* infection

After exposed to *S. enteritidis*, monocytes produced TNF-α and IL-10, as well as macrophages. The TNF-α expression was highest at 24 hours after infection in monocytes (Figure 2A) and macrophages (Figure 2C). IL-10 expression was highest at 72 hours after infection in monocytes (Figure 2B). Whereas in macrophages, HLA-B27$^+$ReA$^+$ macrophages had the highest IL-10 expression level at 72 hours after infection, and HLA-B27$^+$ReA$^-$ as well as HLA-B27$^-$ macrophages showed the highest IL-10 levels at 24 hours after infection (Figure 2D).

HLA-B27$^+$ monocytes produced more TNF-α, but less IL-10 than HLA-B27$^-$ monocytes after 4 hours post-infection (Figure 2A–2D). HLA-B27$^+$/ReA$^+$ macrophages secreted significantly more TNF-α after 4 hours post-infection and less IL-10 than their HLA-B27$^-$/ReA$^-$ counterparts at 24 hours and 72 hours post-infection (Figure 2C, 2D, $p<0.05$ and $p<0.01$, respectively). Also, it is notable that HLA-B27$^+$ReA$^-$ macrophages produced substantially

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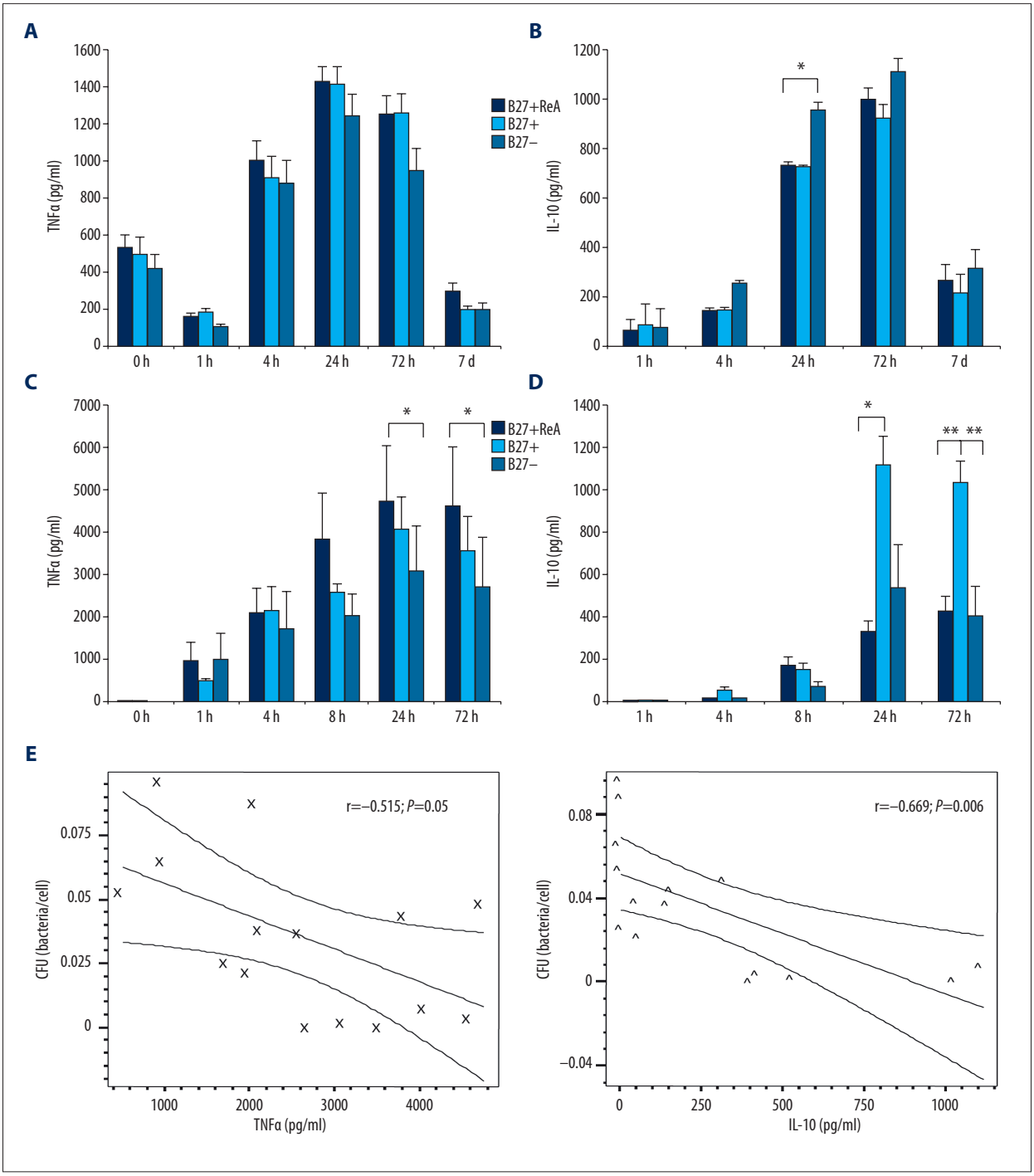

Figure 2. TNF-α and IL-10 production in cells of different groups. The production of TNF-α (**A, C**) and IL-10 (**B, D**) by monocytes (**A, B**) and macrophages (**C, D**) during *S. enteritidis* infection. HLA-B27⁺ cells demonstrated significantly more TNF-α production than HLA-B27⁻ cells. HLA-B27⁺ cells also showed significantly less IL-10 in monocytes but more IL-10 in macrophages. (**E**) The correlation between TNF-α and IL-10 production with the number of intracellular bacteria/CFU. Data were presented as mean value ±SEM. Experiments were done in triplicate and every measurement was done five times, * $p<0.05$, ** $p<0.01$.

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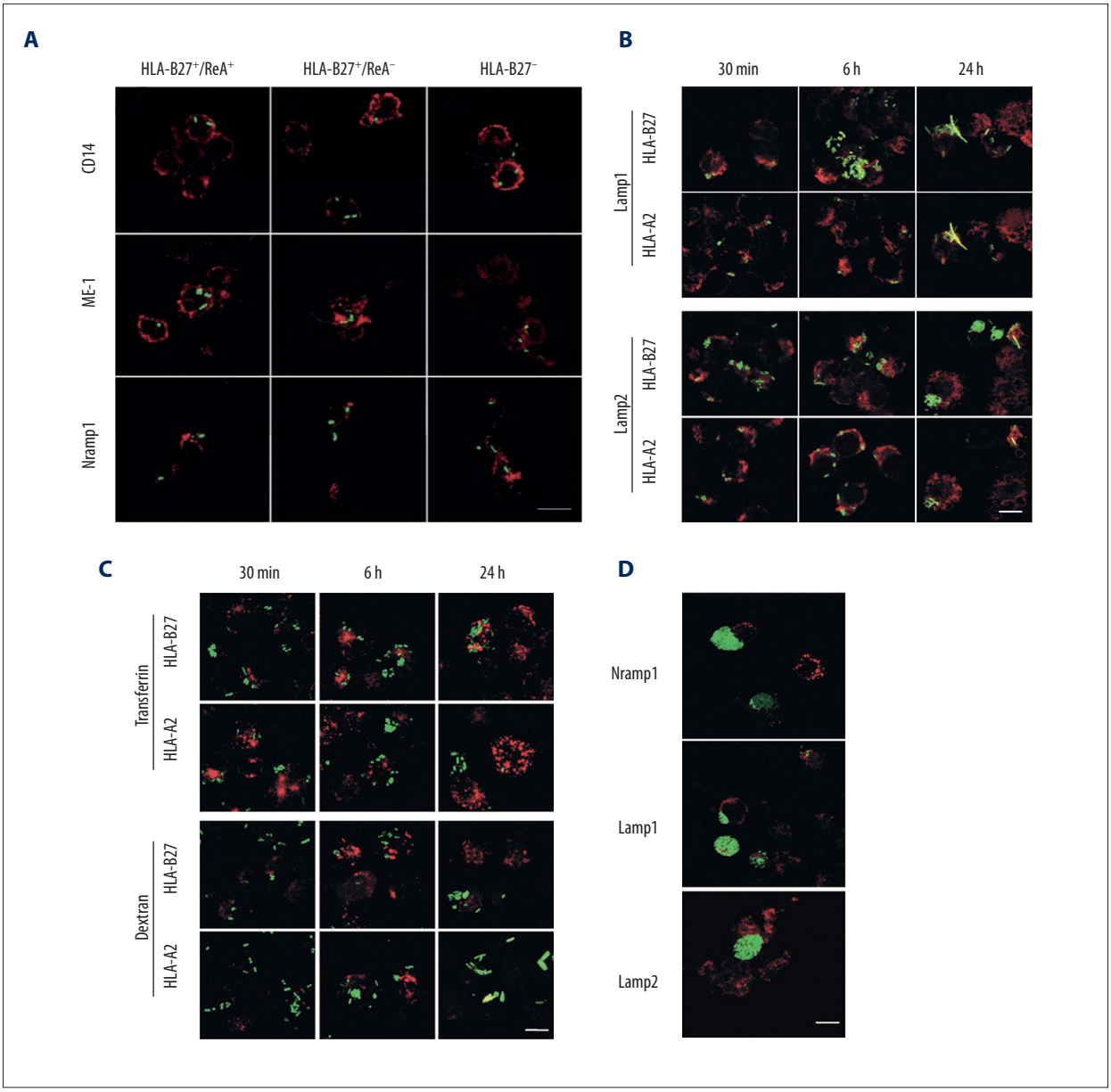

Figure 3. Confocal laser scanning microscopy images of the monocytes from HLA-B27$^+$/ReA$^+$, HLA-B27$^+$/ReA$^-$, and HLA-B27$^-$/ReA$^-$ patients. Monocytes were infected with GFP-*S. enteritidis*. One hour post-infection, monocytes were fixed, permeabilized, and stained with CD14, ME-1 and NRAMP1 antibodies. (**A**) The expression of CD14, ME-1, and NRAMP1 in monocytes of HLA-B27$^+$ReA$^+$, HLA-B27$^+$ReA$^-$, and HLA-B27$^-$ groups. There was no significant difference of the co-localization of intracellular *S. enteritidis* and NRAMP1 between the groups. (**B**) The co-localization of GFP-*S. enteritidis* with lysosomal markers LAMP1/2 in HLA-B27 and HLA-A2 transfected U937 cells. *S. enteritidis* co-localized with LAMP1 and LAMP2 in both HLA-B27 cells and HLA-A2 cells at 30 minutes, 6 hours, and 24 hours post-infection. The cells were stained with LAMP1/2 antibodies. LAMP1/2 (red color) was found co-localized with *S. enteritidis* (green color) in HLA-A2 cells but not seen in HLA-B27 cells. (**C**) Co-localization of GFP-*S. enteritidis* with endocytic trafficking proteins transferrin and dextran. Phagosomes with GFP-*S. enteritidis* did not interact with incoming transferrin or dextran at 30 minutes, 6 hours, and 24 hours either in HLA-B27 or HLA-A2 transfected U937 cells. (**D**) Vacuoles containing many replicated bacteria in HLA-B27 cells did not co-localize with lysosomal markers NRAMP1, LAMP1, or LAMP2. Scale bar: 10 µm.

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more IL-10 than HLA-B27$^+$/ReA$^+$ macrophages at 24 hours and 72 hours after infection (Figure 2D).

The intracellular trafficking of *S. enteritidis*-containing phagosomes

To identify the intracellular bacteria and verify the CFU results, we observed the bacteria-containing cells using a confocal fluorescence microscope. The expression of CD14 and HLA-B27 are similar on the membranes of macrophages from HLA-B27$^+$/ReA$^+$ and HLA-B27$^+$/ReA$^-$ patients. No significant difference was found in the co-localization of bacteria-containing phagosomes and NRAMP1 among the groups (Figure 3A).

We studied the trafficking patterns of bacteria-containing phagosomes in U937 cells using lysosome markers LAMP1 and LAMP2. The co-localization of lysosomal markers and GFP-*S. enteritidis* containing phagosomes were identified by confocal microscopy. Our findings suggested that GFP-*S. enteritidis* containing phagosomes were accessible for the delivery of LAMP1 and LAMP2 in both HLA-B27 cells and HLA-A2 cells. From 30 minutes to 24 hour post-infection, LAMP1 and LAMP2 were detected in more than 60% of the phagosomes containing GFP-*S. enteritidis* (Figure 3B).

We detected transferrin in only 12–17% of *S. enteritidis*-containing vacuoles at 30 minutes, 6 hours, and 24 hours after infection in HLA-B27 and HLA-A2 cells. The co-localization of bacteria-containing phagosomes and endocytic markers was found in less than 20% of the HLA-B27 positive transfected cells and in the control cells at 30 minutes, 6 hours, and 24 hours (Figure 3C). Our results indicated that HLA-B27 did not modulate the interactions between bacteria-containing phagosomes and host endocytic traffic in the infected cells.

The confocal microscopy results demonstrated that the vacuoles containing many replicated bacteria (more than 100 bacteria within a single cell) were found in HLA-B27 cells, and those vacuoles with a large number of replicated bacteria did not co-localize with NRAMP1, LAMP1, or LAMP2 (Figure 3B, 3D). In contrast to HLA-B27 cells, the majority of *S. enteritidis* in HLA-A2 cells were rod-shaped and co-localized with LAMP1/2 (Figure 3B). These results indicated that a plethora of replicated bacteria in HLA-B27 monocytic cells were located within unique vacuoles.

Discussion

The primary findings of this work were that monocytes/macrophages from HLA-B27$^+$/ReA$^+$ patients secreted more TNF-α and less IL-10 than HLA-B27$^-$ReA$^+$ counterparts after *S. enteritidis* infections. HLA-B27 itself was shown to affect the elimination of intracellular *S. enteritidis*, which could sufficiently replicate within HLA-B27$^+$ human monocytic cells but not in HLA-A2 cells. Our results suggested that HLA-B27 could affect the intracellular *S. enteritidis* elimination in monocytes.

Although there have been arguments about the mechanism of the abnormal secretion of TNF-α and IL-10 by monocytes/macrophages in ReA, the roles of TNF-α and IL-10 in intracellular *S. enteritidis* in monocytes/macrophages have been well-documented. Th1 cytokines, including TNF-α, induce cell-mediated-immunity and generate various important pro-inflammatory cytokines to eliminate infectious agents such as bacteria [25,26]. IL-10, belonging to the Th2 cytokine family and has important regulatory functions on immune and inflammatory responses [27]. In addition, it has been reported that several intracellular bacteria, such as *S. enteritidis*, *Mycobacterium*, *Legionella pneumophila*, *Listeria monocytogenes*, and *Leishmania major* could utilize IL-10 for survival in macrophages of mice by disabling intracellular killing [28]. In recent studies, the levels of TNF-α and IL-10 in peripheral blood of acute and chronic ReA have been detected. TNF-α was less expressed in ReA patients than rheumatoid arthritis (RA) patients and healthy persons. In this study, the HLA-B27$^+$ patients secreted less TNF-α than their HLA-B27$^+$ counterparts. Compared with RA patients, chronic ReA patients were shown to have a similar production of TNF-α and lower IL-10, while acute ReA patients showed lower TNF-α but comparable IL-10 level [29]. Activated monocytes/macrophages are the major producers of TNF-α and IL-10. Enhanced TNF-α production has been observed in adhering cultured monocytes from HLA-B27 positive healthy persons with a history of ReA [30,31]. In our *ex vivo* models, HLA-B27$^+$ monocytes and macrophages produced less IL-10 than their HLA-B27$^-$ counterparts upon *S. enteritidis* infection. As showed earlier, PBMCs derived from patients with *S. enteritidis*-trigged ReA expressed elevated cytokines, including TNF-α and IL-10, was compared with patients recovered from *S. enteritidis* infection without arthritis [32]. Similarly, both TNF-α and IL-10 levels in HLA-B27 transfected U937 cells were shown to be elevated upon *S. enteritidis* infection [33]. Using monocytes from peripheral blood of HLA-B27$^+$ healthy persons (with or without ReA history), we provided novel evidences that impaired clearance of intracellular microbes is correlated with the abnormal expression of cytokines. The production of TNF-α and IL-10 by monocyte-derived macrophages was correlated with intracellular bacteria elimination. Taken together, the clearance of intracellular bacteria in monocytes/macrophages might be critical to maintaining the balance of TNF-α and IL-10 production after the bacteria infection. However, TNF-α and IL-10 production was not found to be closely related to intracellular *S. enteritidis* number in monocytes. The detailed reason remains to be clarified.

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For a long time, it has been widely accepted and well elucidated that the function of HLA-B27 is to present arthritogenic peptides in the pathogenesis of ReA. HLA-B27 has a peptide binding specificity and presents self-peptide mimicking pathogen-derived epitopes to the autoreactive CD8+ T lymphocyte (CTL) [34,35]. However, the impaired function of intracellular bacteria elimination in HLA-B27$^+$ monocytes and macrophages was to not found to be caused by an antigen presentation function, suggesting that there could be other uncharacterized functions of HLA-B27. The human U937 monocytic cells transfected with HLA-B27 eliminated *S. enteritidis* and *Yersinia* inefficiently, and activated the NF-κB pathway when stimulated by *S. enteritidis* LPS [36]. This suggests that HLA-B27 plays a non-antigen-presenting role in ReA development. Subsequent research has provided evidence that HLA-B27 disturbed the interaction between monocytic cells and *S. enteritidis*. In addition, the intracellular bacteria number was higher in HLA-B27 cells than in mock cells. The inhibition of bacteria-containing phagosome-lysosome fusion events, and the expression of some natural resistance factors to infection such as NRAMP1 [37,38], are achieved by the modulation of intracellular signaling transduction pathways by HLA-B27. Briefly, the heavy chain of HLA-B27 is susceptible to protein misfold and endoplasmic reticulum (ER) overload, which, therefore, leads to the modulation of intracellular signaling transduction pathways.

In our study, the co-localization of bacteria-containing phagosomes with NRAMP1 was not different among all the groups, indicating that the recruitment of NRAMP1 on the phagosome membranes might not be related to the differences in intracellular bacteria clearance. On the other hand, the monocytes/macrophages from HLA-B27$^+$/ReA$^+$ and HLA-B27$^+$/ReA$^-$ patients showed different profiles in intracellular bacteria elimination and cytokines production. These results indicated that factors other than HLA-B27 exist in the impairment of host defense procedure, and work in the development of ReA in HLA-B27$^+$ individuals. Membrane reporters such as CD14 and toll-like receptors (TLRs) have been suggested to affect the internalization of bacteria [39]. Based on our findings, the impaired elimination of intracellular *S. enteritidis* may not involve membrane CD14 and NRAMP1 expression.

The elimination and killing of intracellular bacteria generally involves either oxygen-dependent or oxygen-independent mechanism. The oxygen-dependent killing mechanism involves the reduction of oxygen to superoxide anion by membrane-bound enzymes, leading to the generation of hydrogen peroxide, hydroxyl radicals, and nitric oxide (NO). The oxygen-independent mechanism, on the other hand, includes several modifications of the bacteria-containing vacuoles on pH, cationic proteins, lysozymes, lactoferrin, etc. Killing intracellular *S. enteritidis* within U937 monocytic cells is independent of NO production [40]. We therefore suspected that the impaired elimination of *S. enteritidis* within HLA-B27 cells could be related to the impaired oxygen-independent killing mechanism.

The trafficking of bacteria is closely associated with their replication within the host cells; therefore we further conducted the intracellular trafficking study to evaluate the bacteria intracellular trafficking patterns within HLA-B27 and HLA-A2 cells. We found that 60% of *S.* enteritidis-containing vacuoles (SCVs) co-localized with lysosomal markers LAMP1 and LAMP2 early, at 30 minutes post-infection, but less than 12% of SCVs were accessible to incoming transferrin and dextran. The *S. typhimurium*-containing vacuoles in epithelial cells have been shown to fuse with compartments containing lysosomal membrane glycoproteins LAMP1 and LAMP2 but avoid certain lysosomal enzymes such as cathepsin D using a unique pathway [41]. SCVs, thus, do not intersect with the endocytic route and bypass an intermediate organelle in lysosome biogenesis [16]. Together with these findings, our results revealed that intracellular pathogens could select a certain type of lysosomes and form a highly-specialized phagosome to survive and replicate. In addition, our confocal microscopy results showed that most of the SCVs with a high number of bacteria were negative for both LAMP1 and LAMP2, indicating the heterogeneity of SCVs within macrophages.

Endosomes can be protected from resident hydrolytic enzyme digestion by LAMP1. Even a partial reduction of LAMP1 levels in these compartments may modify them to promote the intracellular bacteria survival and growth [42,43]. Lin et al. reported that pathogenic *Neisseria* type 2 lgA1 protease from *Neisseria* could increase the rate of LAMP1 degradation, which ultimately promotes bacteria survival within human epithelial cells [44]. Thus, *S. enteritidis* could possibly survive within human macrophages by cleaving LAMP1. The SCVs with a high number of replicated bacteria showed negative or very small amount of LAMP1 on their phagosome membranes, indicating the involvement of the efficient bacteria replication within these SCVs. We thus speculated that the compromised elimination of intracellular bacteria may be due to the impaired inhibition of bacteria replication by HLA-B27, possibly by affecting the bacteria-containing phagosome maturation.

Conclusions

In general, our data suggested that, in persons with a history of ReA, HLA-B27 modulated the intracellular survival of *S. enteritidis* in monocytes and monocyte-derived macrophages. HLA-B27 led to the aberrant production of TNF-α and IL-10 by monocytes/macrophages after *S. enteritidis* infection, indicating its association with ReA pathogenesis. The SCVs could escape the normal degradative pathways in the host cells and allow the bacteria replicate sufficiently.

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