

Original Article

Eperythrozoon suis infection enhances atherosclerosis in low-density lipoprotein receptor-deficient mice

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Abstract: Background: *Eperythrozoon* (EP) infection in human shows high (35-70%) prevalence in northwest China. As this area also has the highest incidence of coronary disease in young people in China, we speculated that there is a possible association between EP infection and atherosclerotic development, which was tested in a murine model. Methods: EPs were isolated from three patients who were confirmed to have *Eperythrozoon suis* infection. Forty 10-week old wild type C57BL/6 and the same number of low-density lipoprotein receptor-deficient (LDLR^{-/-}) C57BL/6 mice were inoculated respectively with human EP 10 times (once a day); and 20 C57BL/6 and the same number of (LDLR^{-/-}) C57BL/6 mice were not injected with EPs as controls. The halves of EP inoculated mice were administered with tetracycline. Atherosclerotic lesions and C5b-9 precipitation in aorta sections were visualized with Oil Red O staining and immunohistology 30 weeks after the last inoculation. *Eperythrozoon suis* antigen, antibody, complements, and cytokines in the blood and blood vessels were measured with ELISA. Results: Atheroma formation was accelerated after inoculation of EP in LDLR^{-/-} mice. The atherosclerotic lesion size was significantly reduced after treatment with tetracycline (P<0.01), proinflammatory cytokine production, C3 turnover, C5b-9 precipitation, and atherosclerotic lesion size remained significantly different in the blood and within the aortas of infected mice compared with controls (P<0.01). Conclusions: EP suis infection activated complements, induced inflammatory cytokine expression, and aggravated atherosclerosis in murine models.

Keywords: *Eperythrozoon suis*, zoonosis, atherosclerosis, complement activation

Introduction

Eperythrozoon (EPs), (also called *hemotropic mycoplasmas* or *hematoplasmas*) are a group of mycoplasmal pathogens that adhere to the surface of red blood cells (RBCs) and are capable of inducing severe anemia. They are the global etiological agents of infectious anemia in a variety of animals, including humans. Since the first human infection case was reported by Puntaric et al. [1] in 1986, many cases have been reported in China [2-4]. The most recent epidemiological survey showed that the prevalence of human EP infection in the Inner Mongolia province of China was approximately 35-70% [5]. In addition, this region shows a high incidence of serious coronary and vascular diseases in individuals under the age of 40 [6], suggesting a possible link between the two conditions.

EP mainly infects RBCs, causing hemolysis, hemorrhage, and intravascular coagulation, but its effects on atherosclerosis remain relatively unexplored. The classic risk factors of atherosclerosis include smoking [7], hypercholesterolemia [8], hypertension [9], and diabetes [10]. However, together, these factors account for only about 50% of atherosclerosis cases. Infectious agents, including *Chlamydia pneumoniae* [11], *Mycoplasma* [12], *Rickettsia* [13], and many viruses [14] have also been suggested as possible risk factors. Therefore, we hypothesized that EP infection might alter lipid metabolism and increase serum cholesterol levels to indirectly promote atherosclerosis. We inoculated murine models with EPs isolated from human patients, assessed whether the infection would cause or aggravate atherosclerosis, and attempted to elucidate the possible mechanisms.

Eperythrozoon aggraves atherosclerosis

Materials and methods

Inoculation preparation

EP samples were obtained from three EP infected patients that were initially diagnosed clinically, and then the presence of EP was confirmed by light microscopy, scanning electronic microscopy [2], and polymerase chain reaction (PCR) and sequencing. After confirmation, blood samples from the patients (5 ml) were collected in EDTA anticoagulation tubes and centrifuged at 5000 g for 10 min at 4°C. The precipitated cell pellets were lysed and washed with 40 ml cold water containing 0.1 mg/ml inosine 5'-triphosphate (Hangzhou Kai Peng Biotechnology Co. Ltd., Hangzhou, China) three times. The purified EP suis particles were counted with an automated cell counter. The concentration of EP was adjusted to 10⁵ cells/ml in mouse serum with 0.1 mg/ml inosine and maintained at -80°C for mouse inoculation.

Mice

A total of 60 wild type C57BL/6 mice and 60 low-density lipoprotein receptor-deficient (LDLR^{-/-}) C57BL/6 mice (SLAC, Shanghai, China) were used. Twenty of the wild type and the same number of LDLR^{-/-} mice were injected with 10 µl normal mouse serum respectively as controls (groups A and D). The other 80 mice were inoculated with 10 µl mouse serum containing about 10⁶ EP particles for 10 times (once a day). The infected wild type and LDLR^{-/-} mice were further divided into two groups: one that received tetracycline administration (group B and E, respectively) and the other without (group C and F, respectively). Tetracycline was dissolved into the drinking water (0.1 mg/ml) beginning from 8 weeks after the last EP inoculation. Since previous experiments showed that several mice died from infection as of 35 to 40 weeks if not treated with tetracycline [15], we chose 30 weeks after inoculation as the end point of the experiment.

Ethics statements

All of the patients were formally informed and signed written consent forms according to the guidelines of the ethics committee of Affiliated Hospital of Inner Mongolia Medical University before giving blood samples. The animal experiments were carried out according to the Guideline for the Use of Animals in Science Act, 12482

2008 (China Scientific Procedures). The project was approved by Ethnic Committee of Inner Mongolia Medical University, reference number YKD2015090.

Microscopic observation of EP infection in human and murine models

Blood smears obtained from patients and the EP inoculated mouse tail were stained with Wright-Giemsa stain (Sigma, Sino-American Biotechnology Company, Beijing, China). The stained blood smears were then observed under a light microscope (Leica, Germany).

Detection of EP infection in human and mice with PCR

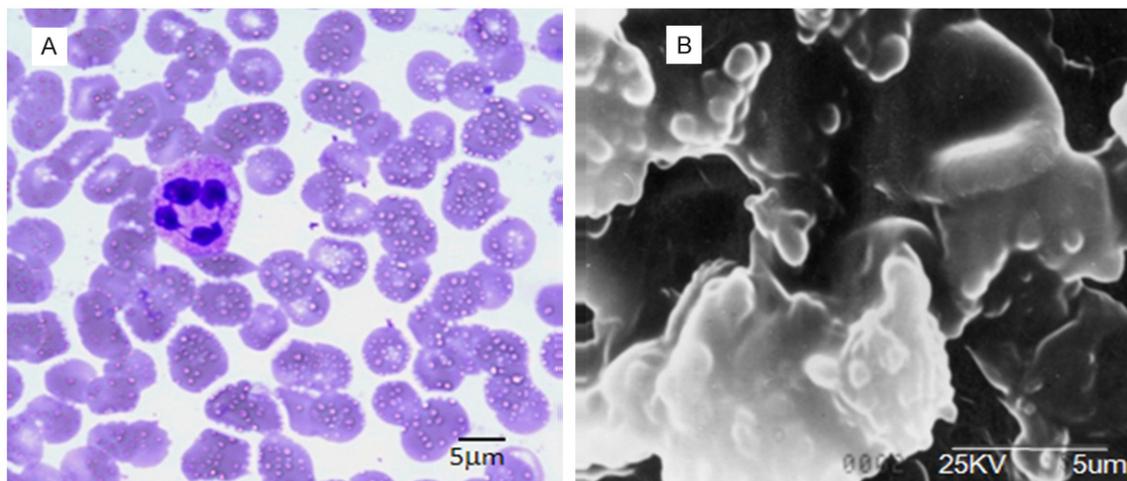
DNA was isolated from the patients' and mouse tail blood using reagent (Qiagen). PCR was performed according to the manufacturer's instructions (Perkin-Elmer) using specifically designed primers targeting the 16S rRNA gene of *Mycoplasma suis* strain KF740480: 3'-GATT-AATGCTGGTGGTATGC-5' and 5'-TAGTTCTAATAAGACTGAAT-3. PCR conditions were 94°C for 4 min and 30 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. The final cycle was 72°C for 7 min. The PCR samples were run on a 1.5% agarose gel containing ethidium bromide, and bands were made visible by UV transillumination. The purified PCR fragments were sequenced with NextSeq500.

Cholesterol and triglyceride analysis

Blood was withdrawn from the inferior vena cava of mice after sacrifice and allowed to clot on ice. Serum was kept at 4°C for up to 24 h before analysis. Serum total cholesterol and triglycerides were measured enzymatically for each mouse using kits (Jian Cheng, Nanjing, China) according to the manufacturer's instructions. Lipoprotein profiles were analyzed from pooled sera by size-exclusion chromatography using a SMART micro-fast-performance liquid chromatography (FPLC) system (Pharmacia, Stockholm, Sweden) (16).

Aortic root histology

Cryosections of the aortic root were taken, according to the published methods [17, 18]. For each mouse, the entire aortic root was serially sectioned into 5-µm sections beginning at the point where the three valve leaflets first appeared, and every 10th section (50 µm) was



C

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Mycoplasma suis strain HLJ/2010 16S ribosomal RNA gene, partial sequence
 Sequence ID: [gb|KF740480.1](#) Length: 1473 Number of Matches: 1

Range 1: 408 to 980 [GenBank](#) [Graphics](#) ▾ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1035 bits(560)	0.0		/573(0%)	Plus/Plus
Query 68	TGCCCGCCCTTGATTGTA	ACTAATTGAATAAGTGACAGCTAACTATGTGCCAGCAGCTGC		127
Sbjct 468	TGCCCGCCCTTGATTGTA	ACTAATTGAATAAGTGACAGCTAACTATGTGCCAGCAGCTGC		527
Query 128	GGTAAACATAGGTCACGAGCATTATCCGGATTTATTGGCCGTA	AAAGGAAGCGTAGGCTG		187
Sbjct 528	GGTAAACATAGGTCACGAGCATTATCCGGATTTATTGGCCGTA	AAAGGAAGCGTAGGCTG		587
Query 188	AAATGTGTATTCATTGTTAAAAATATTTGCTTAACAAGTGTTCCGGTGAAGATTACATT		247	
Sbjct 588	AAATGTGTATTCATTGTTAAAAATATTTGCTTAACAAGTGTTCCGGTGAAGATTACATT		647	
Query 248	TCTAGAATTAGTTAGAGGGTACTGGAATTC AATGTGTAGTGGTGGAAATACGTAGATATAT		307	
Sbjct 648	TCTAGAATTAGTTAGAGGGTACTGGAATTC AATGTGTAGTGGTGGAAATACGTAGATATAT		707	
Query 308	TGAGGAACACCAGAGGCTAAGCGGAGTGCCGGAACATAAATTGACGCTGAGGCTTGA	AAAG	367	
Sbjct 708	TGAGGAACACCAGAGGCTAAGCGGAGTGCCGGAACATAAATTGACGCTGAGGCTTGA	AAAG	767	
Query 368	CGTGGGTAGCAAATGGGATTAGATACCCAGTAGTCCACGCCGTAACCGATGGGTATTAG		427	
Sbjct 768	CGTGGGTAGCAAATGGGATTAGATACCCAGTAGTCCACGCCGTAACCGATGGGTATTAG		827	
Query 428	TCATTTGGATTTAAGACTGAGTGATGTAGCTAACCGGTTAAATACCCCGCTGGGTAGTA		487	
Sbjct 828	TCATTTGGATTTAAGACTGAGTGATGTAGCTAACCGGTTAAATACCCCGCTGGGTAGTA		887	
Query 488	TATATGCAAAATATGAAACTCAAAGAAATTGACGGGGACCTGAACAAGTGGTGGACATGT		547	
Sbjct 888	TATATGCAAAATATGAAACTCAAAGAAATTGACGGGGACCTGAACAAGTGGTGGACATGT		947	

Figure 1. Diagnosis of *Eperythrozoon suis* (EP) infection. A: The blood smear was stained with Wright-Giemsa stain (light microscope, 1000×). B: EP invading RBCs causing deformation (scanning electron microscope, 5600×). Scale bar = 5 µm. C: EP infection confirmed with PCR amplification of 16S rRNA gene of *Eperythrozoon*. The PCR fragments were sequenced and blast query.

stained with Oil Red O and counterstained with Mayer's hematoxylin.

Localization of EP and C5b-9 precipitation on the aorta by immunohistochemistry

Immunohistochemistry for frozen sections was conducted based on the technique described

previously [18]. The sections (10-µm thick) were cut from the aortas of mice, air-dried, and fixed in ethanol. Slides were washed with Tris-buffered saline and 0.3% Tween 20 (TBST, pH 7.4). Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 10 min. Sections were washed 3 times with TBST and blocked with 10% serum in TBST

Table 1. Body weight and total serum lipid levels in control and EP-infected mice

	Group A (WT Control)	Group B (WT, EP+Tet)	Group C (WT, EP)	Group D (LDLR ^{-/-} , Control)	Group E (LDLR ^{-/-} , EP+Tet)	Group F (LDLR ^{-/-} , EP)
N	20	20	20	20	20	19
Final body weight (g)	28.66 ± 3.11	25.64 ± 3.19	26.97 ± 2.97	28.51 ± 3.59	28.11 ± 3.37	29.02 ± 3.29
Total cholesterol (mmol/l)	4.97 ± 0.84	4.36 ± 0.61	4.72 ± 0.66	8.72 ± 1.88**	8.62 ± 1.91**	9.39 ± 1.92**
Total triglyceride (mmol/l)	1.43 ± 0.45	1.39 ± 0.37	1.41 ± 0.42	1.78 ± 0.54*	1.69 ± 0.41*	1.81 ± 0.43*

WT: wild type mice; EP: inoculated with *Eperythrozoon suis*; +Tet: tetracycline treatment. Data presented are mean ± SEM. *P<0.05, **P<0.01.

for 10 min, and then a rabbit antibody against EP (College of Animal Science and Technology, Hebei North University, Hebei, PR China) and a rabbit anti-C5b-9 (Shanghai Ruiqi Biotech Company, Shanghai, China) were added at a 1/100 dilution in TBST. Sections were incubated at room temperature for 1 h in a humidified chamber and washed 3 times with TBST. Horseradish peroxidase-conjugated anti-rabbit antibody (1/100 dilution, DAKO) was used as the secondary antibody, and sections were incubated for 30 min before they were washed 5 times with TBST. Horseradish peroxidase activity was detected with use of a DAB solution (DAKO). The reaction was stopped, and sections were counterstained with Mayer's hemalum, dehydrated, and mounted with DPX (BDH).

Measurement of C3, C3a, and C5b-9 in the blood

The levels of blood complements C3, C3a, and C5b-9 were measured using ELISA kits (Ximei, Shanghai, China). The monoclonal antibodies specific for C3, C3a, and C5b-9 were pre-coated onto microplates. Standards and samples were pipetted into the wells so that any complement elements present would be bound by the immobilized antibodies. Enzyme-linked monoclonal antibodies specific for complement elements were added to the wells. Following a few washes to remove any unbound antibody-enzyme reagents, a substrate solution was added to the wells and the color developed in proportion to the amount of complement elements bound in the initial step was evaluated on a microplate reader.

Quantification of C3, C5b-9 deposition and cytokine production in the aorta

Total protein was isolated from the whole aorta with a kit (Pierce, Sino-American Life Sciences Company, Beijing, China) after en face perfusion staining. The deposition of C3 and C5b-9

in the aortic wall was detected with an ELISA kit (Ximei, Shanghai, China). The production of the cytokines, IL1 β , IL10, and tumor necrosis factor alpha (TNF α) in the aorta was measured using rat anti-mouse cytokine antibodies contained in the specific ELISA kits (Bogu Life Science Company, Shanghai China).

T cell proliferation assay

To investigate the cell-mediated immune response to EP infection, measurement of T cell proliferation was carried out by culturing lymphocytes isolated from the spleen or the para-aortic lymph nodes of infected or control mice in the presence of EP antigen. A total of 2 \times 10⁵ cells/well were seeded in complete culture medium supplemented with 10% fetal calf serum (Gibco, Life Technologies, Invitrogen, Beijing). Cells were stimulated with either 10⁶ EP or phytohemagglutinin (PHA) (5 μ g/ml) (Sigma-Aldrich, Beijing). Cells cultured under similar conditions without any stimulation served as the negative control. The wells were set up in triplicate and incubated for 6 days at 37°C in a 5% CO₂ atmosphere. Sixteen hours before termination of the culture, 1 μ Ci of tritiated (³H) thymidine (Dibai Chemical Ltd., Shanghai, China) was added to each well. The cells were then harvested onto glass fiber filters on a cell harvester and allowed to dry overnight. Two milliliters of scintillation fluid (Sigma-Aldrich Fluka, Sino-American Beijing Bioscience Company, China) was added to each tube containing the dried filter discs and cells were counted using a liquid scintillation beta counter. Cell proliferation was calculated according to the uptake of tritiated thymidine by cells and is expressed as the stimulation index (SI) as follows: mean cpm (test)/mean cpm (control) [19].

Statistical analysis

Values for a given aortic root are expressed as the mean from measurements of five sections. All data are expressed as the mean ± SEM and tested by a two-tailed Student's t-test (Excel

Eperythrozoon aggravates atherosclerosis

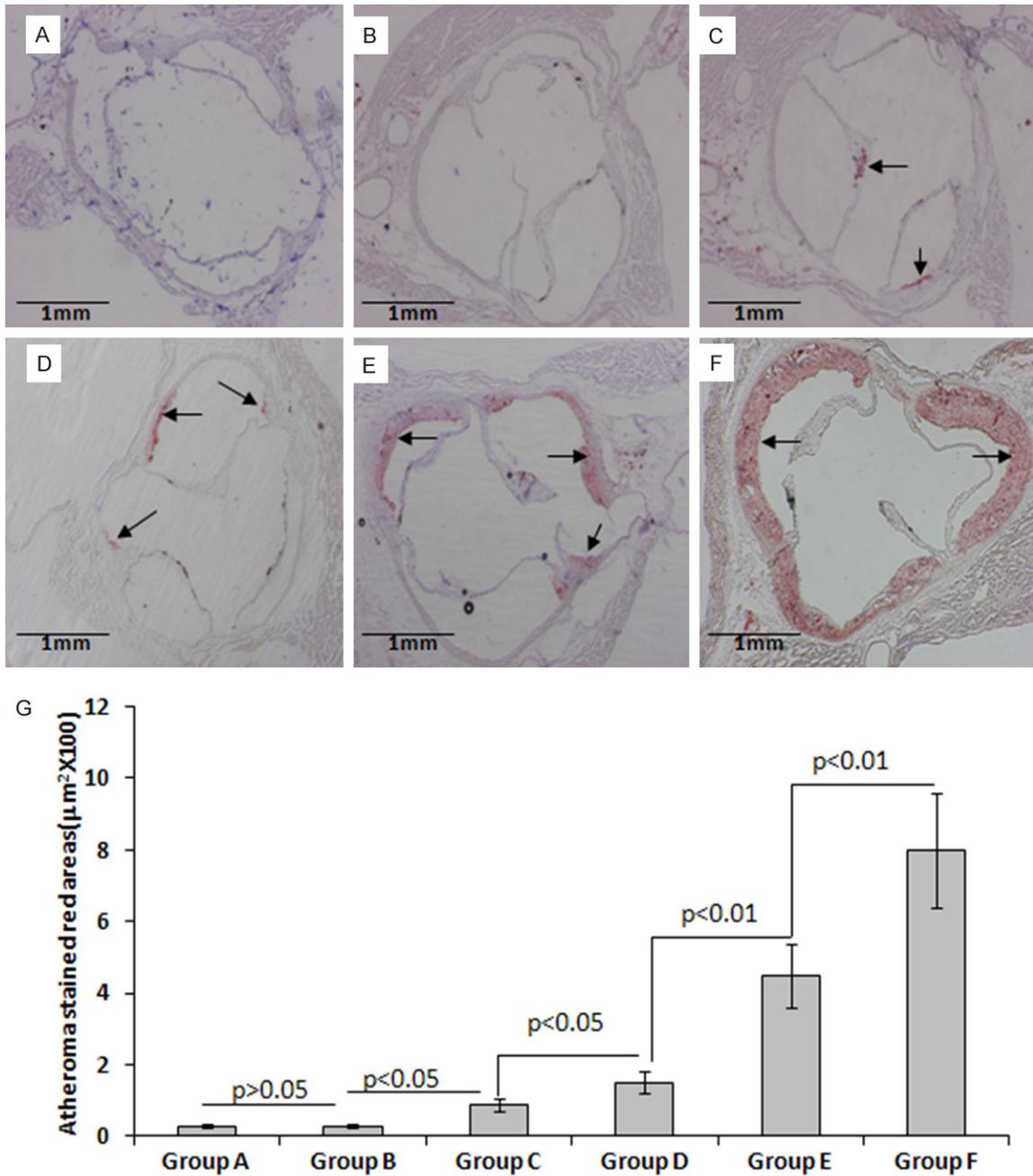


Figure 2. Aortic root sections stained with Oil Red O and counterstained with hematoxylin. A: Wild type control (uninfected); B: Wild type with *Eperythrozoon suis* (EP) infection and tetracycline; C: Wild type with EP infection without tetracycline; D: LDLR^{-/-}-control (uninfected); E: LDLR^{-/-}-with EP infection and tetracycline; F: LDLR^{-/-}-with EP infection without tetracycline; G: Comparison of absolute lesion areas after image analysis of section staining. Values are group means \pm SEM. Arrows indicate atherosclerotic lesions.

and SigmaStat), with significance assumed at $P < 0.05$.

Results

EP infection in human and in inoculated mice

Wright-Giemsa staining of the patient blood smears revealed singular, variably sized myco-12485

plasma attached to the red blood cell membranes, but not to white blood cells', which were observed in clusters centered around a single membrane focus or freely floating in plasma under a light microscope (**Figure 1A**). Scanning electron microscopy showed that the parasite was attached to the RBC membrane surface or invaded the RBC, which caused RBC
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Eperythrozoon aggravates atherosclerosis

Table 2. EP antigen and anti-EP antibody in blood and aortic wall

	Group A (WT control)	Group B (WT, EP+Tet)	Group C (WT, EP)	Group D (LDLR ^{-/-} , Control)	Group E (LDLR ^{-/-} , EP+Tet)	Group F (LDLR ^{-/-} , EP)
N	20	20	20	20	20	19
Blood EP antigen (pg/ml)	7.2 ± 1.1	53.19 ± 5.54	455.44 ± 157.45**	8.3 ± 3.2	59.54 ± 27.13**	495.54 ± 171.22**
Aorta EP antigen (pg/g)	8.2 ± 2.1	9.7 ± 8.2	10.9 ± 4.2	10.1 ± 6.1	8.2 ± 3.3	5.8 ± 2.9
Serum anti-EP antibody (ng/ml)	0.22 ± 0.14	7.64 ± 1.41	19.34 ± 7.22**	0.30 ± 0.23	8.34 ± 1.32**	21.25 ± 12.14**

WT: wild type mice; EP: inoculated with *Eperythrozoon suis*; +Tet: tetracycline treatment. **P<0.01.

Table 3. Complement C3 and C5b-9 levels in the blood and aortic wall

	Group A (WT, Control)	Group B (WT, EP+Tet)	Group C (WT, EP)	Group D (LDLR ^{-/-} , Control)	Group E (LDLR ^{-/-} , EP+Tet)	Group F (LDLR ^{-/-} , EP)
N	20	20	20	20	20	19
Blood C3 (mg/mL)	122.54 ± 19.52	69.71 ± 17.88	47.22 ± 7.84**	106.65 ± 18.42	71.78 ± 19.22**	39.67 ± 9.91**
Aorta C3 (mg/g)	12.12 ± 6.27	14.28 ± 8.15	39.55 ± 10.51*	16.44 ± 7.23	18.22 ± 7.11*	45.17 ± 12.24*
Blood C3a (µg/ml)	1.78 ± 0.24	1.97 ± 0.38	9.25 ± 2.05**	1.17 ± 0.41	2.01 ± 0.29**	12.07 ± 3.27**
Blood C5b-9 (ng/ml)	2.95 ± 5.23	7.77 ± 5.71*	30.17 ± 25.93**	2.15 ± 3.13	44.11 ± 31.22**	100.88 ± 37.33**
Aorta C5b-9 (ng/g)	2.21 ± 4.23	4.94 ± 4.52	17.17 ± 18.93**	2.18 ± 4.15	31.11 ± 20.21**	83.57 ± 47.28**

EP: inoculated with *Eperythrozoon suis*; +Tet: tetracycline treatment. *P<0.05, **P<0.01.

deformation (**Figure 1B**). EP infection was further confirmed by PCR and sequencing. The sequencing data indicated that the infected pathogens of EP 16S rRNA gene was 100% homology to that of EP from porcine (**Figure 1C**). This data confirmed that the pathogen infected human was *Eperythrozoon suis*. The same results were observed in the EP inoculated murine models.

EP infection does not affect body weight or serum lipids

To determine whether differences in lipid metabolism might contribute to EP-induced atherosclerosis, cholesterol and triglyceride levels were examined in all groups. No significant differences in final body weight, total serum cholesterol, or triglyceride levels were observed between the wild type mice with and without EP infection. However, the levels of cholesterol and triglyceride were significantly different between the wild type and LDLR^{-/-} mice (P<0.05; **Table 1**).

EP infection enhances atherosclerotic lesion formation in the aortic roots

The atherosclerotic lesions formation in EP-infected LDLR^{-/-} mice without antibiotic treatment (group F) were most evident in the aortic roots, with larger lesions, either when expressed as the absolute lesion area (P = 0.001) or as a fraction of the aortic root area (P = 0.01), compared with the mice treated with tetracycline (group E) (**Figure 2**). Additionally, the aortas of wild type mice with EP infection

and no tetracycline (group C) showed minor atherosclerotic formation; however, the group with EP infection and tetracycline administration (group B) and the negative control (group A) did not show any atherosclerotic lesions (**Figure 2**).

EP does not infect the aorta

To examine whether EP antigen was present within the vascular wall of infected mice, we harvested the aortas and isolated total proteins at 30 weeks after infection. ELISA was employed to measure the EP antigen level in the aorta. Although the EP antigen concentration in the blood of infected mice without antibiotics (tetracycline) treatment was significantly higher than that of the mice treated with the antibiotics (P<0.001; **Table 2**), the EP antigen level within the aortic wall was similar to that of the background (P>0.05; **Table 2**). Immunohistological staining also confirmed that there was no EP pathogen present in the aortic wall in all groups of mice (data not shown).

EP infection increases C3 turnover and enhances C3 and C5b-9 deposition in the aorta

To examine whether EP infection could activate complements, we assessed the C3 turnover rate, and changes in C3a and C5b-9 in the blood. As expected, the circulating C3 level was reduced by 62-66% in all EP-infected mice (**Table 3**). The blood levels of C3a and C5b-9 significantly increased after infection with EP (P<0.05), which were reduced by 50% and 65%, respectively, with tetracycline treatment (**Table 3**). The data also showed that the increased C3

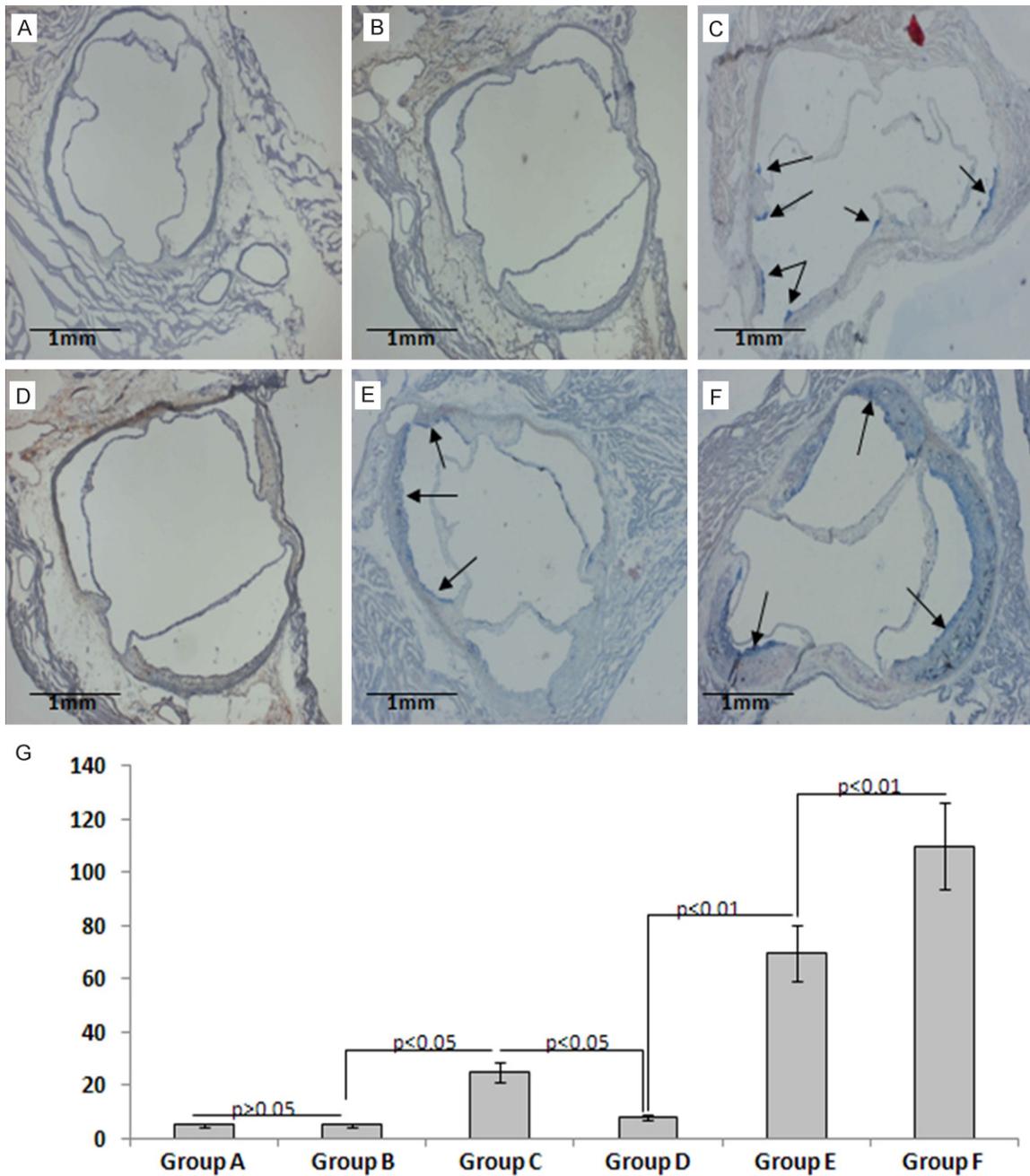


Figure 3. Deposition of C5b-9 in the aortic roots. Photomicrograph of cryosections of aortic roots of wild type (A-C) and LDLR^{-/-} mice (D-F) stained with anti-C5b-9. C5b-9 deposition was seen on lesional areas. (G) shows a semi-quantitative comparison between anti-C5b-9 staining in different groups. Arrows indicate the deposition of C5b-9.

turnover and circulating C3a levels were not associated with the level of serum lipids ($P > 0.05$), but were strongly associated with EP infection status ($P < 0.01$). To examine whether C3 and C5b-9 could be precipitating on the aortic wall, total protein from the aorta was extracted and ELISA was used to measure the complement levels. An abundant amount of C3 was

found in the aortic wall of EP-infected mice. After treatment with tetracycline, the precipitation of C3 was reduced by 62.5-63.7% (Table 3).

As shown in Table 3, EP infection induced C5b-9 accumulation in blood and on blood vessels. The C5b-9 levels differed significantly

Table 4. Cytokine production within the aortic wall

	Group A (WT, control)	Group B (WT, EP+Tet)	Group C (WT, EP)	Group D (LDLR ^{-/-} , control)	Group E (LDLR ^{-/-} , EP+Tet)	Group F (LDLR ^{-/-} , EP)
N	20	20	20	20	20	19
IL1β (ng/g)	19.6±37.98	16.21±4.52	93.33±8.34*	21.27±8.12	39.77±15.35**	189.77±45.35**
TNFα (pg/g)	309.44±127.54	315.68±123.43	789.87±219.56**	378.67±159.45	419.78±193.16**	811.67±276.56**
IL10 (pg/g)	144.10±37.43	167.11±58.34	296.38±92.21*	176.55±73.87	173.34±57.17*	272.32±87.34*

Comparison between groups A, B, and C; and between groups D-F, respectively, *p<0.05, **p<0.001.

between the infected *LDLR*^{-/-} groups with and without tetracycline (groups E and F), and both infected groups had significantly higher ($P<0.01$) C5b-9 levels than the control (group D). Similarly, C5b-9 accumulation was significantly higher in the EP-infected wild type mice (group C) compared with the negative control (group A) and tetracycline-treated infected mice (group B; $P<0.05$).

C5b-9 staining in aortic roots of EP infected mice was identified particularly around atherosclerotic lesions (Figure 3C, 3E, 3F). The semi quantitative percentage of aortic root lesions with positive C5b-9 staining was significantly higher in EP infected wild type and *LDLR*^{-/-} mice without antibiotic treatment group compared with the mice treated with tetracycline ($P<0.05$ and <0.01) (Figure 3G).

Immune response of EP-infected mice

To examine whether the development of atherosclerosis in EP-infected mice might be due to an inflammatory immune response, anti-EP antibody in the serum, and the production of the cytokines IL-1β, IL-10, and TNFα in the aorta were measured using ELISA. Compared to groups A and D, after repeated injections of EP, the level of anti-EP antibody (IgM) in the mice of groups C and F increased by 87-fold and 71-fold, respectively, and by 34-fold and 27-fold in the mice administered tetracycline in groups B and E, respectively (Table 2). Thus, although inoculation of EP could induce specific anti-EP antibody production, the antibody did not prevent against further infection in the mouse models. The production of the pro-inflammatory cytokines IL-1β and TNFα increased significantly after EP inoculation without tetracycline treatment ($P<0.01$). However, expression of the anti-inflammatory cytokine IL-10 within the blood vessel wall also increased after EP infection ($P<0.05$). Tetracycline treatment reduced all cytokine production levels ($P<0.05$ or <0.01)

(Table 4). These data indicate that EP infection enhances cytokine production within the aorta, but a high level of serum lipids does not.

To investigate the cellular immune interactions between atherosclerosis and EP infection, T-cell proliferation assays showed no significant differences between the groups of T cells co-cultured with and without EP antigen in the media with or without PHA stimulation (Figure 4, $P > 0.05$).

Discussion

A link between pathogen infection and atherosclerosis has been observed, but the direct and/or indirect influences of other known risk factors remain unclear. Through studies with atherosclerosis models, various stimuli have been suggested to induce inflammatory changes within the arterial wall, which could initiate atherosclerosis either indirectly or directly [20-22]. To establish whether any pathogen is a direct causative agent of atherosclerosis, confirmation from serological studies and the presence of the pathogens in atherosclerotic lesions is required.

One possible explanation for how EP might initiate atherosclerosis is through infection of not only RBCs [23-25] but also vascular endothelial cells, which results in damage to the cell membrane [26]. However, in the present study, immunohistology showed that there was no pathogen present in the aortic wall in the EP-infected mice, indicating that EP does not damage the vascular endothelial cells directly. Together, these data indicate that EP infection is an indirect causative factor of atherosclerotic lesions. This finding was further supported by the fact that EP infection promoted severe atherosclerotic lesion formation under a condition of hyperlipidemia.

We originally proposed that EP infection might act by altering lipid metabolism and increasing

Eperythrozoon aggravates atherosclerosis

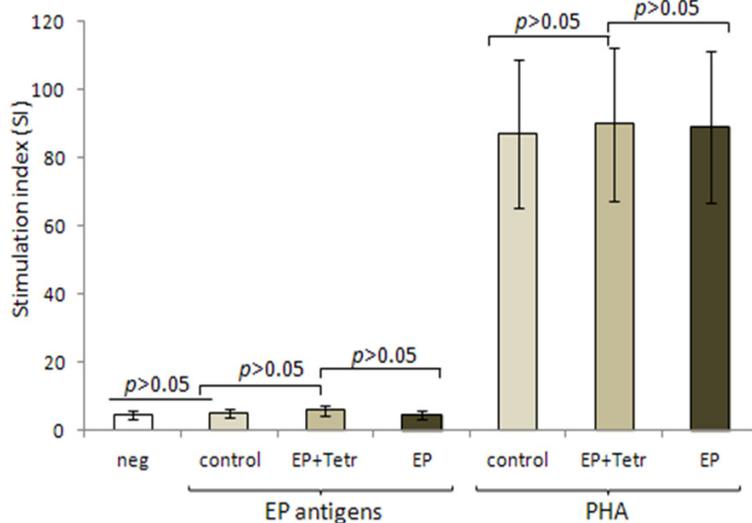


Figure 4. T cell proliferation in the presence or absence of *Eperythrozoon suis* antigen. Neg: medium only; control: T cells only; EP+Tetr: T cells co-culture with EP antigen in the presence of tetracycline; EP: T cells co-culture with EP antigen in the absence of tetracycline.

serum cholesterol levels to promote atherosclerosis indirectly. However, here, the pathogen had no effects on total lipid levels or the lipid profile in a more subtle manner. On the other hand, our study showed that only mice with the coexistence of EP infection and a high level of serum cholesterol developed severe atherosclerosis. EP infection alone was sufficient to induce abnormal complement activation and damage to the vascular endothelium, and caused minor atherosclerosis. Therefore, it seems that a high level of total serum cholesterol is a prerequisite for EP to initiate severe atheroma formation. Although no EP antigen was found within the aortic wall of the mouse models, atherosclerotic lesion formation was reduced by antibiotics administration, indicating that EP indirectly contributes to the development of atherosclerosis.

Our detection of C5b-9 in aortic root lesions of EP infected *LDLR*^{-/-} mice provides the first direct evidence for terminal complement pathway activation in a mouse atherosclerosis model. C5b-9 deposition was significantly reduced in tetracycline treated mice, consistent with a major role of the classic pathway in EP driving C5b-9 generation. Furthermore, C5b-9 deposition was not completely abolished with administration of tetracycline suggesting EP infection induced complement activation

very early, even before starting antibiotics treatment.

Based on our data, we speculate an indirect mechanism by which EP induces expression of a pathogen-specific antibody and forms an antigen-antibody complex, which activates complement elements. In order to test this hypothesis, we measured the complement system in infected mice. We found that the EP antibody and C3 turnover increased after EP inoculation. Moreover, the level of precipitating C5b-9 increased on the blood vessel wall. C5b-9 precipitation on the blood vessel wall may attract white blood cells and produce proinflammatory cytokines, which might

cause damage to the vascular endothelial cell membrane and aggravate inflammation in the vascular wall. These findings suggest that EP infection results in inappropriate complement activation and induces damage to blood cells and the vascular endothelial cell membrane. The detection of C5b-9 in the aortic root lesions of mice provided direct evidence for terminal complement pathway activation in the mouse atherosclerosis model, which is consistent with published work in this model [18]. Although EP infection alone was sufficient to induce abnormal complement activation, caused by C5b-9 deposition on the vascular wall and damaged the vascular endothelium, it was still not sufficient to induce severe atherosclerotic lesion formation.

Normally, wild type C57BL/6 mice can survive for approximately 2 to 2.5 years. Although we tried to raise the mice as long as possible, unfortunately, many EP-infected mice without tetracycline treatment died by 35 weeks after inoculation. Therefore, we chose 30 weeks after EP inoculation as the end point of the experiment.

From a clinical point of view, the results of the present study may add valuable information to our understanding of the role of EP infection in cardiovascular disease. People in the pastoral areas of northwest China are in close contact

with many different kinds of livestock or wild animals. This frequent exposure combined with poor hygienic conditions increases the risk of EP infection for this population. Additionally, their general preference of a high-fat diet suggests that the coexistence of hyperlipidemia and EP infection could increase the risk of atherosclerosis. This may, at least partially, explain the fact that the populations in the northwest areas of China show the highest prevalence of development of atherosclerosis at a young age in the country.

Thus, our study suggests that vascular endothelial injury caused by abnormal complement activation is a risk factor and acts in concert with hyperlipidemia to exacerbate atherosclerosis.

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Disclosure of conflict of interest

None.

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Eperythrozoon aggraves atherosclerosis

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