SUPPLEMENTARY INFORMATION

*Ahnak*-knockout mice show susceptibility to *Bartonella henselae* infection because of CD4+ T cell inactivation and decreased cytokine secretion

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Running Title: *Ahnak* deletion promotes *B. henselae* infection
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Figure S1. Immunohistochemistry for CD4, IFN-γ and TNF-α in the liver tissues. (A) IHC for CD4, CW. (B) IHC for CD4, CK. (C) IHC for CD4, BW (D) IHC for CD4, BK. (E) IHC for IFN-γ, BW. (F) IHC for IFN-γ, BK. (G) IHC for TNF-α, BW. (H) IHC for TNF-α, BK. arrowhead: positive cells. (Original magnification: 400×, scale bar: 50 μm) (I) IHC score for CD4, IFN-γ, and TNF-α. *p < 0.05 according to the Mann–Whitney U test. IHC: immunohistochemistry, CW: control wild-type mice not infected with B. henselae, CK: control Ahnak-KO mice not infected with B. henselae, BW: wild-type mice infected with B. henselae, BK: Ahnak-KO mice infected with B. henselae, IFN-γ: interferon-γ, TNF-α: tumor necrosis factor-α
Figure S2. Flow cytometric analysis profiles and gating strategies (A) CD4+IFN-γ+ cells 
(B) CD4+IL-4+ cells. CW: control wild-type mice not infected with *B. henselae*, CK: control Ahnak-KO mice not infected with *B. henselae*, BW: wild-type mice infected with *B. henselae*, BK: Ahnak-KO mice infected with *B. henselae*
Figure S3. Cytokine levels in the serum samples of the mice infected with or without B. henselae The serum samples were assayed for IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-10, MCP-1, RANTES, TNF-α, and VEGF levels by using the Luminex multiplex murine cytokine analysis kit. †p < 0.05 according to the Mann–Whitney U test. B. henselae: Bartonella henselae, CW: control wild-type mice not infected with B. henselae, CK: control Ahnak-KO mice not infected with B. henselae, BW: wild-type mice infected with B. henselae, BK: Ahnak-KO mice infected with B. henselae, IFN-γ: interferon-γ, IL: interleukin, KO: knockout, MCP-1: monocyte chemoattractant protein-1, RANTES: regulated on activation, normal T cell expressed and secreted, TNF-α: tumor necrosis factor-α, and VEGF: vascular endothelial growth factor.
To determine whether *B. henselae* infection induced any changes in the liver function and serum proteins of the infected mice, ALT, AST, total protein, and albumin levels were measured in the serum samples of the mice obtained during autopsy. *p < 0.05* according to the Mann–Whitney *U* test; †*p < 0.05* according to the Kruskal–Wallis test.

ALT: alanine aminotransferase, AST: aspartate transaminase, *B. henselae*: Bartonella henselae, KO: knockout, CW: control wild-type mice not infected with *B. henselae*, CK: control Ahnak-KO mice not infected with *B. henselae*, BW: wild-type mice infected with *B. henselae*, BK: Ahnak-KO mice infected with *B. henselae*.

**Figure S4. Serum chemistry** To determine whether *B. henselae* infection induced any changes in the liver function and serum proteins of the infected mice, ALT, AST, total protein, and albumin levels were measured in the serum samples of the mice obtained during autopsy. *p < 0.05* according to the Mann–Whitney *U* test; †*p < 0.05* according to the Kruskal–Wallis test.

ALT: alanine aminotransferase, AST: aspartate transaminase, *B. henselae*: Bartonella henselae, KO: knockout, CW: control wild-type mice not infected with *B. henselae*, CK: control Ahnak-KO mice not infected with *B. henselae*, BW: wild-type mice infected with *B. henselae*, BK: Ahnak-KO mice infected with *B. henselae*. 
SUPPLEMENTARY MATERIALS AND METHODS

All animals were used for each experiment (CW group: n = 3, CK group: n = 3, BW group: n = 6, BK group: n = 6). All data are expressed as box-and-whisker plots, the ends of the box represent the upper and lower quartiles, so the entire box spans the interquartile range. The median is marked by a vertical line inside the box. Whiskers represent the maximum and the minimum, the X mark is the mean and the O mark is the outlier. In the box plot, the whiskers are generally defined as 1.5 times the inter-quartile range, and anything outside the whiskers is considered to be an outlier.

Detection of B. henselae DNA in the liver tissues of the mice

*B. henselae* DNA was detected in the mouse liver tissue by performing polymerase chain reaction (PCR) to amplify a part of the *Bartonella* adhesion A (*badA*) gene by using specific primers (sense primer: 5'-GAGAGGATCCTCGAATCTTGCGCTTACAGGA-3' and antisense primer: 5'-GAGACTCGAGTGCTTTTAGCTGTGCAACATT-3'). DNA was extracted from the liver tissues of the mice in all the groups by using QIAamp tissue kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. The same amount of template (1 μg of DNA) from each mouse was used for PCR to amplify a part of the *badA* gene. PCR was performed with *Taq* polymerase (TaKaRa, Tokyo, Japan) in a 25 μl reaction mixture by using the following conditions: initial denaturation at 94°C for 5 min; 35 cycles of amplification with the *badA*-specific primers at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min; and final extension at 72°C for 10 min. *B. henselae* DNA was used as a positive control. The reaction product was visualized by electrophoresing on a 1% agarose gel and by staining with ethidium bromide. The expected product size was 1,007 bp. The density of *badA* PCR band was quantified with Image J (NIH, USA).
Flow cytometric analysis of spleen cells

Spleenocytes were harvested from the mice in all the study groups, washed with PBS, and suspended in fluorescence-activated cell sorter (FACS) washing buffer (0.5% fetal bovine serum in PBS). The cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4°C, followed by staining with phycoerythrin (PE)-conjugated anti-CD19 antibody, fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody, and peridinin chlorophyll protein (PerCP)-conjugated anti-CD8 antibody (PharMingen) for 30 min at 4°C. The stained cells were then analyzed using FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Intracellular cytokine staining

The spleen cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4°C, followed by staining with the FITC-conjugated anti-CD4 antibody for 30 min at 4°C. Spleen cells stained with an appropriate isotype-matched Ig were used as negative controls. Next, the spleen cells were fixed and permeabilized with Cytofix/Cytoperm kit (BD Pharmingen), according to manufacturer’s instructions. Intracellular IL-4 and IFN-γ were stained with fluorescein R-PE-conjugated antibodies (PharMingen, San Diego, CA, USA) in a permeation buffer. Finally, the spleen cells were analyzed using the FACSCalibur flow cytometer with CellQuest program (BD Biosciences).

ELISA for determining the levels of multiple cytokines in spleen cell culture supernatants and serum samples

The spleens were removed from the mice in all the groups on day 18. Spleen cells obtained from each mouse were washed twice with PBS, and their numbers were adjusted to 10^6 viable cells/ml of complete endotoxin-free RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Boehringer Mannheim, Australia), 100 U/ml
penicillin, and 100 μg/ml streptomycin. Next, 5 × 10⁵ cells/well were cultured with or without *B. henselae* (multiplicity of infection [MOI]: 10; [5 × 10⁶ CFU/well]) in 96-well plates at 37°C in a humidified atmosphere containing 5% CO₂. After 3 days, culture supernatants were collected and stored at -70°C until further use. The supernatants were assayed for IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES), TNF-α, and vascular endothelial growth factor (VEGF) levels by using Luminex multiplex murine cytokine analysis kit (Milliplex® MAP Kit; Millipore, Bedford, MA, USA). Serum samples obtained from the mice were also assayed using the same Luminex multiplex murine cytokine analysis kit.

**Histopathological analysis of the liver tissues of the mice**

The liver tissues of the mice were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned into 4-μm-thick sections, and stained with hematoxylin and eosin (H&E). The *B. henselae*-infected cell number was graded using a score of 1 to 6 in five random high-power fields (400×): 1–9, score 1; 10–19, score 2; 20–29: score 3; 30–39: score 4; 40–49: score 5; and 50–59: score 6.

Immunohistochemistry for CD4, IFN-γ, and TNF-α was performed as follows: the paraffin-embedded sections were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to 0.01 M phosphate-buffered saline (PBS, pH 7.4). Subsequently, the sections were subjected to heat-induced epitope retrieval (HIER) using Tris-EDTA, pH9.0 (ThermoScientific, Rockford, I, USA) for 15 min to unmask antigen epitopes followed by cooling at room temperature. Then, endogenous peroxidase blocking was performed by incubating the slides with 3% hydrogen peroxide in methanol for 15 min at room temperature. After washing with PBS buffer, the sections were treated with normal goat serum (S-1000, Vector Labs, Peterborough, UK) for 30 min at room temperature to prevent nonspecific binding of the
primary antibody. Subsequently, the sections were incubated with anti-CD4 (1/100, ab51312, Abcam, Cambridge, UK), anti-IFN-γ antibodies (1/100; 250708, Abbiotec, San Diego, USA) or anti-TNF-α (1/200, ab6671, Abcam) overnight at 4°C. After washing with PBS, the immune complexes were detected using the Dako REAL™ EnVision™ kit (Dako, Glostrup, Denmark). The sections were lightly counterstained with Mayer’s hematoxylin for 30 seconds before dehydration and mounting were performed. The intensity of IHC staining was graded on a scale of 0 (none) to 4 (great intensity).

**Relative quantification of cytokine mRNA by using RNA extracted from the liver tissues**

Relative quantification of cytokine mRNA was performed to estimate the expression of multiple cytokine-encoding genes. Total ribonucleic acid (RNA) was extracted from the liver tissues of the mice by using easy-BLUE™ (iNtron, Sungnam, Korea), according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized using 5 μg RNA and Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer’s instructions. PCR was performed using 1 μl cDNA as the template, except for β-actin gene PCR in which 0.5 μl cDNA was used as the template. Cytokine gene-specific primer pairs were prepared, and PCR was performed using a protocol described by De Boer et al. [1]. Briefly, amplification was performed using a PCR thermal cycler (TaKaRa, Tokyo, Japan) programmed for mouse IL-4, IL-10, and β-actin genes by using the following conditions: one cycle at 94°C for 5 min; 32 cycles (22 cycles for the β-actin gene) at 94°C for 1 min, 56°C for 1 min and 30 s, and 72°C for 2 min; and one cycle at 72°C for 10 min. PCR for the mouse IFN-γ and TNF-α genes was performed using the following conditions: once cycle at 94°C for 5 min; 32 cycles at 94°C for 1 min, 59°C for 2 min, and 72°C for 3 min; and one cycle at 72°C.
for 10 min. Each PCR product (10 μl) was analyzed by performing electrophoresis on a 2% agarose/ethidium bromide gel and was visualized under ultraviolet illumination. The amount of DNA was quantified as band intensities by using GelDoc software (Bio-Rad, Hercules, CA, USA). The relative amount of messenger RNA (mRNA) of each cytokine gene is expressed as the ratio of the mRNA of each cytokine gene to that of the β-actin gene.

**Serum chemistry**

To determine whether *B. henselae* infection induced any changes in the liver function and serum proteins of the mice, alanine aminotransferase (ALT), aspartate transaminase (AST), total protein, and albumin levels were measured in the serum samples obtained from the mice during autopsy with DRI-CHEM 3000 colorimetric analyzer (Fujifilm, Tokyo, Japan).

**SUPPLEMENTARY DISCUSSION**

From the results of the spleen cells (flow cytometry and cytokine levels), Ahnak-KO mice showed an impairment in Th1 and Th2-mediated immunity, although cytokine secretion from macrophages was intact.

Thus, in Ahnak-KO mice, the systemically attenuated Th1 effector T cell response led to severe infection by *B. henselae* in the liver. It is believed that the IFN-gamma mRNA expression was relatively increased due to a greater number of *B. henselae* in the liver of Ahnak KO mice as compared to that in wild-type mice. The degree of *B. henselae* infection was more severe in the BK group, and hence, the infiltration of mononuclear cells such as CD4+ T cells in the liver was much higher in the BK group than that in the BW group. In the immunohistochemistry analyses for CD4, IFN-γ, and TNF-α using liver tissue, the total numbers of CD4+ cells, IFN-γ+ cells, and TNF-α+ cells were higher in the BK group as compared to the BW group.
However, the IHC scores for CD4 (mean ± standard deviation, BW: 2.5 ± 1.3 vs. BK: 3.4 ± 0.9) and IFN-γ (BW: 2.3 ± 0.6 vs. BK: 3.2 ± 0.8) did not reach statistical significance. The IHC score for TNF-α in the BK group was significantly higher than that in the BW group (BW: 2.0 ± 1.0 vs. BK: 3.6 ± 0.5). The representative IHC pictures for CD4, IFN-γ, and TNF-α are presented in Fig. S1. Therefore, it is thought that there was a difference between systemic and local immunity.

As C57BL/6 mice are Th1-dominated, they are relatively resistant to intracellular pathogenic infections. If Ahnak KO mice of BALB/c mice (susceptible background mice) are produced and used, it would be much prudent to study the intracellular pathogenic infections and one could obtain a highly susceptible model for *B. henselae* infection.

**SUPPLEMENTARY REFERENCE**

1. De Boer EC, Rooijakkers SJ, Schamhart DH and Kurth KH (2003) Cytokine gene expression in a mouse model: the first instillations with viable bacillus Calmette-Guerin determine the succeeding Th1 response. J Urol 170, 2004-2008