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

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The present study evaluated the role of AHNAK in *Bartonella henselae* infection. Mice were intraperitoneally inoculated with 2×10^8 colony-forming units of *B. henselae* Houston-1 on day 0 and subsequently on day 10. Blood and tissue samples of the mice were collected 8 days after the final *B. henselae* injection. *B. henselae* infection in the liver of *Ahnak*-knockout and wild-type mice was confirmed by performing polymerase chain reaction, with *Bartonella adhesion A* as a marker. The proportion of *B. henselae*-infected cells increased in the liver of the *Ahnak*-knockout mice. Granulomatous lesions, inflammatory cytokine levels, and liver enzyme levels were also higher in the liver of the *Ahnak*-knockout mice than in the liver of the wild-type mice, indicating that *Ahnak* deletion accelerated *B. henselae* infection. The proportion of CD4+interferon-γ (IFN-γ)$^+$ and CD4$^+$interleukin (IL)-4$^+$ cells was significantly lower in the *B. henselae*-infected *Ahnak*-knockout mice than in the *B. henselae*-infected wild-type mice. In vitro stimulation with *B. henselae* significantly increased IFN-γ and IL-4 secretion in the splenocytes obtained from the *B. henselae*-infected wild-type mice, but did not increase IFN-γ and IL-4 secretion in the splenocytes obtained from the *B. henselae*-infected *Ahnak*-KO mice. In contrast, IL-1α, IL-1β, IL-6, IL-10, RANTES, and tumor necrosis factor-α secretion was significantly elevated in the splenocytes obtained from both *B. henselae*-infected wild-type and *Ahnak*-knockout mice. These results indicate that *Ahnak* deletion promotes *B. henselae* infection. Impaired IFN-γ and IL-4 secretion in the *Ahnak*-knockout mice suggests the impairment of Th1 and Th2 immunity in these mice. [BMB Reports 2019; 52(4): 289-294]

INTRODUCTION

Cat scratch disease (CSD), which is commonly transmitted by scratches or bites of cats or kittens (1, 2), is a zoonosis affecting people worldwide and is caused by *Bartonella henselae* (*B. henselae*) or possibly by *B. clarridgeiae*. CSD caused by *B. henselae* usually manifests regional lymphadenopathy in immunocompetent individuals; however, bacillary peliosis hepatis, bacillary splenitis and bacillary angiomatosis are also found in immunocompromised individuals (3, 4).

The prognosis of CSD is generally excellent even in debilitating patients. CSD in healthy individuals usually resolves spontaneously within months without permanent sequelae. However, it may be potentially life threatening for an individual with immunocompromised.

B. henselae is an intracellular pathogen; therefore, immunity against *B. henselae* is mediated by Th1 cells in immunocompetent hosts (3, 5). Interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) play a key role in *B. henselae* elimination (3, 6). In *B. henselae*-infected cats, Th2 cells are predominantly activated and result in the incomplete elimination of *B. henselae* (2). Therefore, these cats show prolonged *B. henselae* infection and act as reservoirs (7, 8).

AHNAK is the largest protein on our body and involved in the formation of cytoskeletal structure, muscular regeneration, and calcium homeostasis (9) and is involved in several biological processes. We previously reported that AHNAK is

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involved in obesity and cellular adipogenesis (10-12). Moreover, AHNAK functions as a tumor suppressor protein to prevent the development of breast and lung cancers by inhibiting cancer cell growth through the potentiation of transforming growth factor-β (TGF-β) signaling pathway (13, 14). Immunologically, AHNAK is an essential component of calcium signaling during CD4+ T cell activation (15). Matza *et al*. reported that *Ahnak*-knockout (KO) mice showed impaired CD4+ T cell proliferation and decreased interleukin (IL)-2 production upon *in vitro* stimulation with an anti-CD3 antibody (15). However, the role of AHNAK in immune regulation and infections has not been completely understood. Therefore, we evaluated the immune responses of *Ahnak*-KO mice to *B. henselae* infection to elucidate whether these mice could be used as an animal model for CSD.

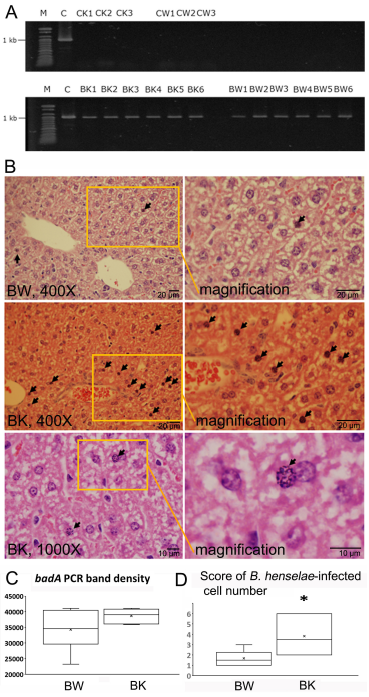

Fig. 1. Detection of *badA* in the liver tissues of the *B. henselae*-infected mice PCR & liver tissues of the *B. henselae*-infected mice. (A) PCR was performed to amplify a part of *badA* with the specific primers. The reaction product was visualized by electrophoresing; M: 100-bp DNA size marker and C: control (DNA from *B. henselae*; *badA* PCR product size: 1,007 bp; template DNA: 10 μg). (B) Hematoxylin and eosin staining: Magnification of the marked area (yellow) shown in right panel. (C) *badA* PCR band density (D) Score of *B. henselae*-infected cell number. *P < 0.05 according to the Mann-Whitney *U* test. *badA*: *Bartonella* adhesion A, *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*.

RESULTS

The four experimental groups were as follows: (i) control wild-type mice not infected with *B. henselae* (CW group), (ii) control *Ahnak*-KO mice not infected with *B. henselae* (CK group), (iii) wild-type mice infected with *B. henselae* (BW group), and (iv) *Ahnak*-KO mice infected with *B. henselae* (BK group).

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

PCR of *badA* indicated the presence of *B. henselae* DNA in the liver tissues of the mice in the BW and BK groups but not in the liver tissues of the mice in the CW and CK groups (Fig. 1A). *badA* PCR band density was not significantly different between the BW and BK groups (Fig. 1C).

Histopathological analysis of the liver tissues of the mice

B. henselae-induced histopathological changes were determined in the mice in the different study groups. A few *B. henselae*-infected cells were detected in the liver tissues of the mice in the BW group (Fig. 1B, D). However, many *B. henselae*-infected cells were detected in the liver tissues of the mice in the BK group (Mann-Whitney *U* test, P = 0.026; Fig. 1B, D). Granulomatous mononuclear cell infiltration was detected in the liver tissues of the *B. henselae*-infected mice on day 18 after initial infection with 2×10^8 *B. henselae*, and the degree of this infiltration was significantly more severe in the mice in the BK group than in those in the BW group (Fig. 2A, B). Immunohistopathology for CD4, IFN-γ and TNF-α in the liver

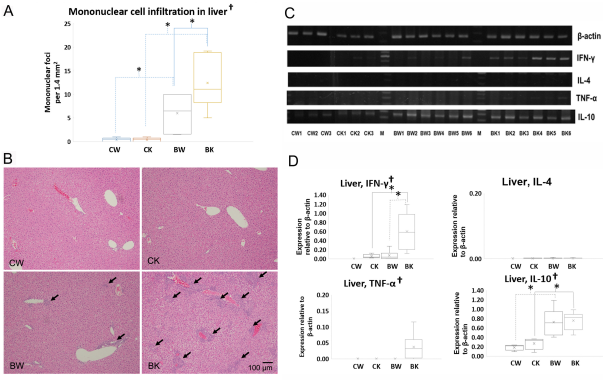

Fig. 2. Mononuclear cell infiltration in the liver & relative quantification of cytokine mRNA by using RNA extracted from the liver tissues. (A) Mononuclear cell infiltration in the liver (mononuclear foci per 1.4 mm^2). (B) Representative liver sections of the mice in the CW, CK, BW, and BK groups. (C) PCR amplification band. (D) Gene expression relative to the β-actin gene expression. *P < 0.05 according to the Mann-Whitney *U* test; †P < 0.05 according to the Kruskal-Wallis test. *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*.

tissues are presented in Fig. S1.

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

A significant difference was observed in the mRNA expression of the IFN-γ, TNF-α, and IL-10 genes relative to that of the β-actin gene in the liver tissues of the mice in the four study groups (Kruskal-Wallis test, P = 0.008, P = 0.023, and P = 0.009, respectively; Fig. 2C, D). The mRNA expression of the target genes was not significantly different between the liver tissues of the mice in the CW and CK groups. However, the mRNA expression of the IFN-γ gene was significantly higher in the mice in the BK group than in those in the BW group. The mRNA expression of the IL-10 gene was significantly higher in the mice in the BW group than in those in the CW group (Mann-Whitney *U* test, P = 0.024). Moreover, the mRNA expression of the IFN-γ and IL-10 genes was significantly higher in the mice in the BK group than in those in the CK group (P = 0.024 and P = 0.024, respectively).

Flow cytometric analysis of spleen cells

The proportion of B cells was not statistically significant among the groups (Fig. 3A). The proportion of CD4+ and CD8+ cells was significantly different among the mice in the different groups (Kruskal-Wallis test, P = 0.019 and P = 0.025, respectively). The proportion of CD4+ cells was significantly higher in the mice in the BK group than in those in the BW group (Mann-Whitney *U* test, P = 0.015; Fig. 3B). The proportion of CD8+ cells was significantly lower in the mice in the BK group than in those in the CK group (Mann-Whitney *U* test, P = 0.024; Fig. 3C). The proportion of IFN-γ+CD4+ and IL-4+CD4+ cells was significantly different among the mice in the different groups (Kruskal-Wallis test, P = 0.006 and P = 0.013, respectively). The proportion of IFN-γ+CD4+ cells was significantly lower in the mice in the BK group than in those in the BW group (Mann-Whitney *U* test, P = 0.004; Fig. 3D). The proportion of IL-4+CD4+ cells was also significantly lower in the mice in the BK group than in those in the BW group (Mann-Whitney *U* test, P = 0.026; Fig. 3E). Flow cytometric analysis profiles and gating strategies

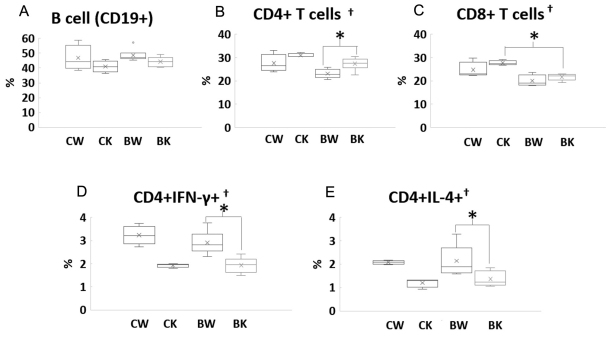

Fig. 3. Flow cytometric analysis of spleen cells. (A) The proportion of B cells, (B) the proportion of CD4+ T cells, (C) the proportion of CD8+ T cells, (D) the production of IFN-γ in CD4+ T cells, and (E) the production of IL-4 in CD4+ T cells. *P < 0.05 according to the Mann-Whitney *U* test; †P < 0.05 according to the Kruskal-Wallis test. 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*.

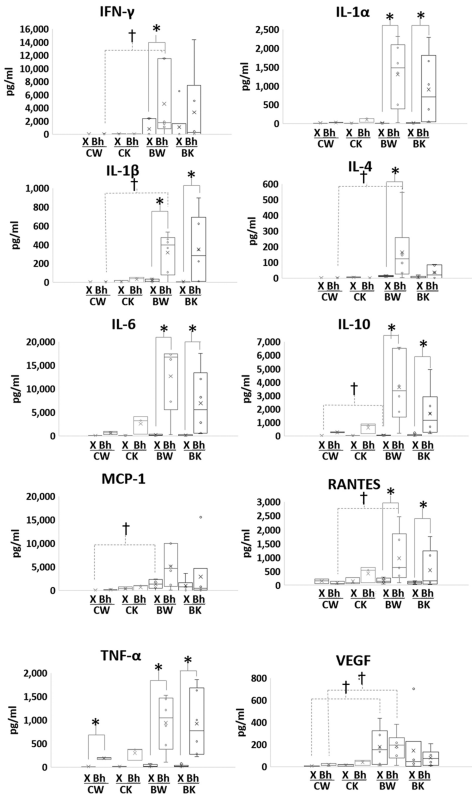

Fig. 4. Cytokine levels in culture supernatants of spleen cells. In all, 5 × 10^5 spleen cells/well were cultured in the presence or absence of *B. henselae* (MOI: 10 [5 × 10^6 CFU/well]) in 96-well plates at 37°C in a humidified atmosphere containing 5% CO_2. After 3 days, the culture supernatants were collected and stored at −70°C for further analysis. The supernatants 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 Wilcoxon signed-rank test; †P < 0.05 according to the Mann-Whitney *U* test. *B. henselae*: *Bartonella henselae*, Bh: *B. henselae*-stimulated spleen 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*, IFN-γ: interferon-γ, IL: interleukin, KO: knockout, MCP-1: monocyte chemoattractant protein-1, MOI: multiplicity of infection, RANTES: regulated on activation, normal T cell expressed and secreted, TNF-α: tumor necrosis factor-α, VEGF: vascular endothelial growth factor, and X: unstimulated spleen cells.

of Fig. 3C and D are presented in Fig. S2.

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

IFN-γ and IL-4 levels were significantly higher in the culture supernatant of spleen cells obtained from the mice in the BW group and cultured in the presence of *B. henselae* than in that of spleen cells obtained from these mice and cultured in the absence of *B. henselae* (Wilcoxon signed-rank test, P = 0.043 and P = 0.028, respectively; Fig. 4). However, IFN-γ and IL-4 levels were not significantly different in the culture supernatants of spleen cells obtained from the mice in the BK group and cultured in the presence or absence of *B. henselae* (P = 0.068 and P = 0.068, respectively). IL-1α, IL-1β, IL-6, IL-10, RANTES, and TNF-α levels were significantly higher in the culture supernatants of spleen cells obtained from the mice in the BW and BK groups and cultured in the presence of *B. henselae* than in those of spleen cells of obtained from these mice and cultured in the absence of *B. henselae*. However, MCP-1 and VEGF levels were not significantly different between the culture supernatants of spleen cells obtained from the mice in the BW and BK groups and cultured in the presence of *B. henselae* than those of spleen cells obtained from these mice cultured in the absence of *B. henselae*. Furthermore, IFN-γ, IL-1β, IL-4, RANTES, and VEGF levels were significantly higher in the culture supernatant of spleen cells obtained from the mice in the BW group and cultured in the presence of *B. henselae* than in that of spleen cells obtained from the mice in the CW group and cultured in the presence of *B. henselae* (Mann-Whitney *U* test, P = 0.048, P = 0.048, P = 0.024, P = 0.048, and P = 0.048, respectively; Fig. 4). However, no significant difference was observed in the levels of these cytokines between the culture supernatants of spleen cells obtained from the mice in the CK and BK groups and cultured in the presence of *B. henselae*.

IL-10, MCP-1, and VEGF levels were significantly higher in the culture supernatant of spleen cells obtained from the mice in the BW group and cultured in the absence of *B. henselae* than in that of spleen cells obtained from the mice the CW group and cultured in the absence of *B. henselae* (Mann-Whitney *U* test, P = 0.048, P = 0.048, and P = 0.048, respectively; Fig. 4). However, no significant difference was observed in the levels of these cytokines between the culture supernatants of spleen cells obtained from the mice in the CK and BK groups and cultured in the absence of *B. henselae*.

TNF-α level was significantly different in the serum samples of the mice in the different groups (Kruskal-Wallis test, P = 0.046). The highest IFN-γ, IL-4, IL-6, IL-10, MCP-1, RANTES, TNF-α, and VEGF levels were detected in the serum samples of the mice in the BW group. However, the increase in the levels of these cytokines was not statistically significant, except for the increase in TNF-α level. TNF-α level was significantly higher in the serum samples of the mice in the BK group than in those of the mice in the CK group. However, no significant differences were observed in the levels of other cytokines between the serum samples of the mice in the CW and BW groups (Fig. S3).

Serum chemistry

ALT, AST, and globulin levels were significantly higher and albumin levels were significantly lower in the mice in the BK group than in those in the CK group (Fig. S4). ALT levels were significantly higher in the mice in the BW group than in those in the CW group.

DISCUSSION

A previous study reported that the liver was the most exclusively affected organ in *B. henselae*-infected C57BL/6 mice and that cultivatable organisms cleared in the spleen and liver homogenates of C57BL/6 mice 6 days after *B. henselae* inoculation (4). *BadA* is a trimeric autotransporter adhesion molecule expressed by *B. henselae* and is implicated in *B. henselae* infection (16). Therefore, we used *badA* as a marker to determine *B. henselae* infection in the liver tissues of the *Ahnak*-KO and wild-type mice. The number of *B. henselae*-infected cells increased in the liver tissues of the *Ahnak*-KO mice. Granulomatous lesions and inflammatory cytokine levels were higher in the liver tissues of the *Ahnak*-KO mice than in those of the wild-type mice. Moreover, *B. henselae* infection significantly increased liver enzyme levels, indicating that *B. henselae* infection induced slight liver damage. Moreover, the degree of the increase in the liver enzyme levels was higher in the *Ahnak*-KO mice than in the wild-type mice. Total globulin levels increase in the presence of an infection, chronic inflammation, low albumin levels, and other disorders. Therefore, the result obtained for globulin levels in the present study indicate higher degree of *B. henselae* infection in the *Ahnak*-KO mice than in the wild-type mice.

The Th phenotype of CD4+ T cells involved in infectious agent-induced cell-mediated immunity can be differentiated based on the cytokine secretion pattern of these cells (17); Th1 cells have a role in controlling intracellular pathogen infection by IL-2 and IFN-γ secretion, and macrophage activation induction. In contrast, Th2 cells have a role in helping B cells to elicit antibodies by IL-4, IL-5, and IL-13 secretion (18). Because *B. henselae* is an intracellular pathogen, immunity against this pathogen is mediated by Th1 cells in immunocompetent hosts (3, 5). *B. henselae* infection elicits a Th1-type immune response in humans and mice, which is characterized by increased IFN-γ and TNF-α secretion and subsequent nitric oxide production. Cellular immune responses are primarily mediated by CD4+ T cells and interfere with *B. henselae* pathogenesis. In the present study, we observed that *B. henselae*-induced systemic Th1 immune response decreased in the absence of *Ahnak* expression. Moreover, we observed that IFN-γ secretion was significantly elevated in the spleen cells obtained from the *B.*

henselae-infected wild-type mice but was not elevated in the spleen cells obtained from the *B. henselae*-infected *Ahnak*-KO mice. *B. henselae* infection also significantly increased IL-4 secretion from Th2 cells in the culture supernatant of spleen cells obtained from the wild-type mice. Similar to IFN-γ, IL-4 production was not significantly increased in the spleen cells obtained from the *Ahnak*-KO mice infected with *B. henselae*. In contrast, TNF-α, IL-1β, and VEGF secretion from macrophages was not significantly different in spleen cells cultures in response to *B. henselae* infection irrespective of *Ahnak* deletion.

Calcium plays important roles in T cell activation and proliferation and cytokine production (19, 20). Matza *et al.* reported that 50% reduction in Cav1.1α1 membrane protein expression in TCR-stimulated $Ahnak^{-/-}$ T cells in the presence of IL-2 and AHNAK is critical for Cav1 channel-mediated calcium signaling in T cells (15). In the study by Matza *et al.*, *Ahnak*-KO mice showed severely impaired IFN-γ production, which is required for *Leishmania major* clearance *in vivo*, and a dramatic increase in IL-4 production, suggesting that AHNAK is required for the induction of Th1 response against *L. major* (15). The pathogenesis of *B. henselae* infection is very similar with that of *L. major* infection because both *B. henselae* and *L. major* are intracellular pathogens. Therefore, we expected that the *Ahnak*-KO mice would show a severe impairment in IFN-γ production and a dramatic increase in IL-4 production in response to *B. henselae* infection and would show the same immune response as that observed in *B. henselae*-infected cats. However, in the present study, the *Ahnak*-KO mice showed an impairment of both IFN-γ and IL-4 production, suggesting the impairment of Th1 and Th2 immunity. Thus, the results of the present study suggest the potential of the *Ahnak*-KO mice to be used as a mild immunocompromised animal model or susceptible animal model for *B. henselae* infection.

MATERIALS AND METHODS

Experimental animals

This study included nine wild-type and nine *Ahnak*-KO C57BL/6 female mice, which were generated in our previous study (10, 21, 22). All the mice were aged 10-11 weeks at the time of use in the study. This study was reviewed and approved by the Institutional Animal Care and Use Committee of the Samsung Biomedical Research Institute (SBRI). SBRI is an Association for the Assessment and Accreditation of Laboratory Animal Care International accredited facility and abides by the Institute of Laboratory Animal Resources guidelines. All procedures used in this study were in compliance with the Animal Welfare Act Regulations and the Guide for the Care and Use of Laboratory Animals.

Experimental groups and inoculation of the mice with *B. henselae*

The mice were divided into the following four groups: (i) control wild-type mice not infected with *B. henselae* (CW group; n = 3), (ii) control *Ahnak*-KO mice not infected with *B. henselae* (CK group; n = 3), (iii) wild-type mice infected with *B. henselae* (BW group; n = 6), and (iv) *Ahnak*-KO mice infected with *B. henselae* (BK group; n = 6). The mice in the BW and BK groups were intraperitoneally inoculated with 100 μl bacterial suspension containing 2×10^8 colony-forming units (CFU) of *B. henselae* Houston-1 (ATCC 49882, American Type Culture Collection, Manassas, VA) suspended in phosphate-buffered saline (PBS) on day 0 and subsequently on day 10. The mice in the CW and CK groups were intraperitoneally injected with 100 μl saline on the same days. Blood and tissue samples of the mice were collected on day 8 after the final *B. henselae* injection (day 18 of the study).

Detailed procedures of experimental methods are described in the supplementary information.

Statistical analysis

All data are expressed as box-and-whisker plots. Data were analyzed using Kruskal-Wallis test followed by Mann-Whitney *U* test. Wilcoxon signed-rank test was used to compare means from two related samples. Confidence levels of 95% or higher were considered significant ($P < 0.05$). All statistical analyses were performed using SPSS software version 21.0 (IBM, Armonk, NY, USA).

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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