Veterinary Microbiology 237 (2019) 108402

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Chemokine receptor 4 (CXCR4) blockade enhances resistance to bacterial internalization in RAW264.7 cells and AMD3100, a CXCR4 antagonist, attenuates susceptibility to *Brucella abortus* 544 infection in a murine model

Alisha Wehdnesday Bernardo Reyes, Lauren Togonon Arayan, Tran Xuan Ngoc Huy, Son Hai Vu, Chang Keun Kang, Wongi Min, Hu Jang Lee, John Hwa Lee, Suk Kim*

Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Jinju, 52828, Republic of Korea

ARTICLE INFO

Keywords:
AMD3100
B. abortus
Cytokines
CXCR4
Invasion
MAPKs

ABSTRACT

We investigated the involvement of chemokine receptor type 4 (CXCR4) signaling on the outcome of *Brucella* (*B.*) *abortus* 544 infection in murine macrophages and in a mouse model. CXCR4 manipulation were first evaluated for *Brucella* invasion and intracellular survival efficiency, mitogen-activated protein kinases (ERK1/2, JNK, p38α) activation and generation of nitric oxide (NO), and then in the splenic bacterial proliferation and cytokine production in BALB/c mice. CXCR4 blockade is involved in the successful control of *Brucella* invasion, reduction of ERK1/2 phosphorylation and inhibition of nitric oxide release from macrophages. Furthermore, using a reported CXCR4-specific antagonist AMD3100 resulted in splenomegaly but attenuated *Brucella* proliferation in these organs with elevated serum levels of MCP-1, TNF and IL-12. These findings provide insights on the contribution of CXCR4 signaling in the phagocytic pathway and immune modulation during *B. abortus* infection.

1. Introduction

Brucellosis is a zoonotic bacterial disease with 500,000 reported human cases annually in endemic regions and causes reduced fertility, abortion, poor weight gain and marked reduction in milk yield in infected animals leading to devastating losses to livestock industry and small-scale livestock holders (Khan and Zahoor, 2018; Franc et al., 2018). *B. abortus* is one of the most common species of *Brucella* that was first reported as a causative agent of premature delivery in cattle and intermittent fever in humans (Khan and Zahoor, 2018). Unlike other pathogens, *B. abortus* is an intracellular organism that does not produce exotoxins, cytolytic enzymes, antiphagocytic capsules, toxic lipopolysaccharides (LPS), thick cell walls, resistance forms, fimbriae or plasmids but can invade and replicate in host professional and non-professional phagocytic cells and its long latent period makes early diagnosis after infection impossible (Cha et al., 2012). The virulence of *Brucella* depends on its capacity to bind to distinct phagocytic or unknown receptors, which is one of the initial and crucial stages of infectious process, and subsequently invade different host cells (Reyes et al., 2017a, b). *Brucella* establishes successful strategies for intracellular survival, being inaccessible to the host humoral immune response, and often inhibit the normal function of the host such as inhibition of apoptosis, modification of membrane-bound vesicles and interruption of phagosome-lysosome formation (Jung et al., 2018).

Microbial infection is detected by pattern-recognition receptors (PRRs) to induce innate immune responses for pathogen control and *Brucella* is known to invade a variety of cell types among which macrophages play a central role as the first line of defense as well as the primary target of this pathogen (Jung et al., 2018; Hajishengallis et al., 2008). Macrophages are known to express chemokine CXC-type receptor 4 (CXCR4)-a cell surface protein that belongs to the seven transmembrane domain G-protein-coupled receptor family which is directly involved in a number of biological processes including immune response (Busillo and Benovic, 2007; Triantafilou et al., 2008). CXCR4 has been reported to be involved in LPS binding, triggers signaling in response to LPS, serves as a TLR2-associated receptor, participates in TLR4 signaling, and independently promotes migration of antigen presenting cells (APCs) outside of blood vessels and into secondary lymphatic organs (Triantafilou et al., 2008; Kishore et al., 2005; Nagashima et al., 2017a). Interaction with CXCR4 has been implicated as a mechanism of immune evasion in *Porphyromonas gingivalis* infection in vitro and in vivo (Hajishengallis et al., 2008). Furthermore, T-trophic HIV-1 virus was inhibited by LPS possibly due to competition for the same receptor CXCR4 (Verani et al., 2002). Given the reported

* Corresponding author.
E-mail address: kimsuk@gnu.ac.kr (S. Kim).

https://doi.org/10.1016/j.vetmic.2019.108402
Received 13 July 2019; Received in revised form 27 August 2019; Accepted 28 August 2019

contributions of CXCR4 signaling in favor of the pathogen and to the best of our knowledge it has never been reported to have any role in brucellosis, hence we investigated its involvement in the invasion of *B. abortus* in macrophages as well its proliferation in a murine model.

2. Materials and methods

2.1. Reagents

Purified anti-mouse CXCR4 antibody was purchased from BioLegend Inc., CA, USA. Mouse CXCR4 siRNA, silencer negative control no.1 siRNA and Lipofectamine RNAiMAX were obtained from ThermoFisher Scientific, MA, USA. LPS, AMD3100 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich, MO, USA. Rabbit polyclonal anti-p-ERK1/2, anti-p-JNK, anti-p-p38, anti-ERK1/2, anti-JNK, anti-p38 and anti-β actin antibodies were purchased from Cell Signaling Technology, Inc., MA, USA. BD cytometric bead array (CBA) mouse inflammation kit was purchased from BD Biosciences, CA, USA. Griess reagent was purchased from Promega, WI, USA.

2.2. Cell culture and bacterial strain

Murine macrophage RAW 264.7 cells were obtained from American Type Culture Collection (VA, USA) and maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2 atmosphere overnight. A smooth, virulent *B. abortus* 544 biovar 1 strain (ATCC 23,448) was routinely cultured using Brucella broth at 37 °C with vigorous shaking until stationary phase.

2.3. Cell transfection

Overnight culture of RAW 264.7 cells were transfected for 24 h with siRNA targeting CXCR4 in parallel with a negative control siRNA using Lipofectamine RNAiMAX according to manufacturer's instructions. The knockdown efficiency was determined by comparing the expression of CXCR4 at the mRNA level in cells transfected with CXCR4 siRNA and control siRNA using reverse transcription (RT)-PCR.

2.4. Cell viability assay

RAW 264.7 cells (1×10^5 cells per well) were cultured in 96-well plates overnight and incubated with CXCR4-neutralizing antibody (1 μg/ml), CXCR4 siRNA (10 μM) or AMD3100 (0, 0.25, 0.5, 1, 2.5, 5, 10, 25 μg/ml) for 48 h with an appropriate control. Dilution of CXCR4-neutralizing antibody or AMD3100 was performed using PBS. Cell viability was determined using MTT assay and the highest non-cytotoxic concentration of AMD3100 was used in the succeeding experiments.

2.5. RNA isolation and RT-PCR

Total RNA of transfected RAW264.7 cells in 6-well plates was isolated using RNAeasy Plus Mini Kit (Qiagen, Hilden, Germany) and 1 μg of RNA was reverse-transcribed into cDNA using QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplification reactions were carried out in a MyGenie96 Thermal Block thermal cycler (Bioneer, Daejeon, Republic of Korea) using the PCR conditions and the oligonucleotide sequences reported by Tissir et al. (2004). PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

2.6. Bactericidal assay

Brucella (2×10^4 colony forming units (CFU)/ml) was incubated with AMD3100 in 96-well plates and incubated at 37 °C for 24 h. Each sample was serially diluted, plated on Brucella agar and incubated at 37 °C for 4 days. CFU was determined and bacterial survival rates were calculated as previously described (Reyes et al., 2017a).

2.7. Bacterial infection assay

Transfected (1×10^4 cells per well) or pre-treated macrophages in 96-well plates (1×10^5 cells per well) were used for bacterial internalization and intracellular survival efficiency determination as previously described (Reyes et al., 2017a).

2.8. Nitrite detection assay

Transfected (1×10^4 cells per well) or pre-treated RAW 264.7 cells in 96-well plates (1×10^5 cells per well) were infected with *B. abortus* at multiplicity of infection (MOI) of 100 for 1 h and incubated in fresh medium with or without CXCR4-neutralizing antibody or AMD3100 for 24 or 48 h. Nitrite accumulation in the culture medium was measured using Griess reagent according to the manufacturer's instructions.

2.9. Western blot analysis

Transfected RAW 264.7 cells (1×10^6 cells per well) in 6-well plates were infected with *B. abortus* for 30 min. Lysing, protein concentration measurement, SDS-PAGE and western blot analysis for MAPKs were done as previously described (Reyes et al., 2017b). The blots were quantified using NIH ImageJ software.

2.10. Animal experiment

All animal procedures were performed in compliance with established federal guidelines and institutional policies by the Animal Ethical Committee of Chonbuk National University (Authorization Number CBNU-2018-101). Female pathogen-free BALB/c mice (8–10 weeks old; Samtako Bio Co. Ltd., Osan, Korea) were acclimatized prior to experiment. The animals were randomly grouped into two non-infected groups pretreated intraperitoneally (i.p.) with AMD3100 (25 μg in 0.1 ml of PBS) or PBS (0.1 ml; negative control), and two infected groups pretreated i.p. with AMD3100 or PBS (positive control). Infection was done i.p. with *B. abortus* 544 (2×10^4 CFU in 0.1 ml PBS) after 24 h and AMD3100 or PBS treatment was repeated at 6 and 9 days p.i. At 3 days p.i., blood was collected via tail vein. Mice were sacrificed at 14 days p.i., blood was collected from the heart and the spleens were removed, weighed and a part were homogenized in PBS. Serial dilutions of homogenized spleens were plated onto Brucella agar and incubated at 37 °C for 4 days.

2.11. Flow cytometry

Serum samples from mice were collected to measure several important cytokines involved in the outcome of *Brucella* infection including IL-6, IL-10, MCP-1, IFN-γ, TNF and IL-12p70 according to the manufacturer's instructions using FACSCalibur flow cytometer (BD Biosciences, CA, USA) in combination with a representative data acquisition and analysis package (BD CellQuest™ software).

2.12. ELISA

IL-1β levels from cell culture supernatants and mouse serum samples were determined by ELISA (abcam, MA, USA) while serum alanine aminotransferase 1 (ALT) concentration was determined using an ALT (Mouse) ELISA kit (BioVision Inc., CA, USA) according to the manufacturers' instructions.

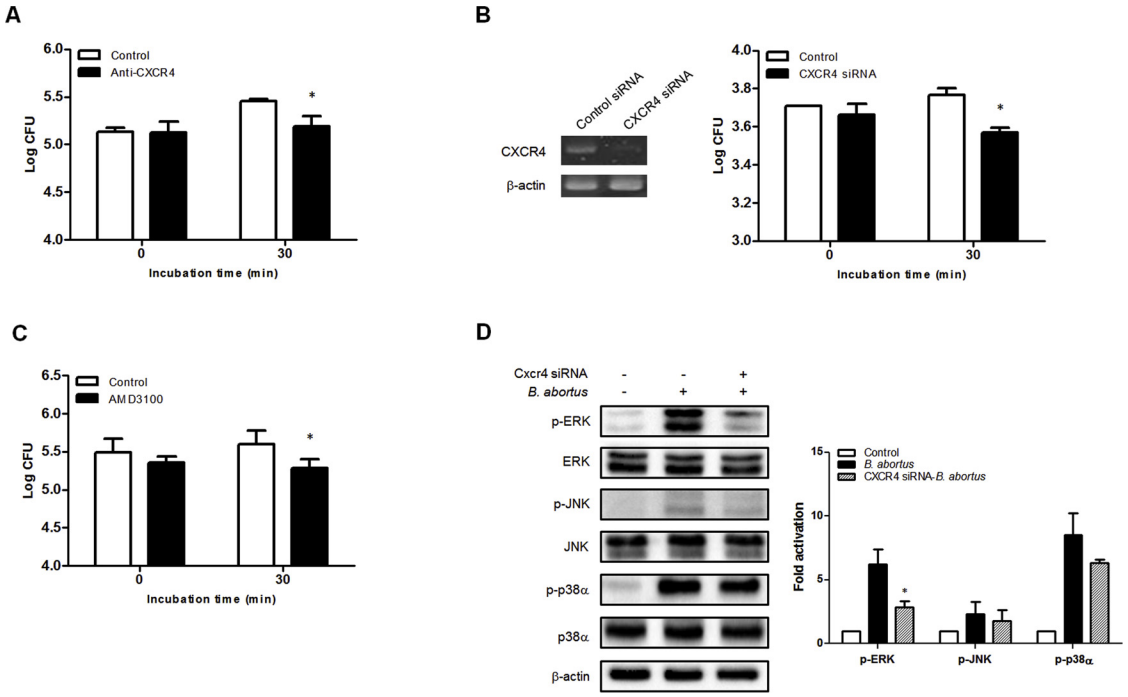

Fig. 1. Effects of CXCR4 blockade on the uptake of *B. abortus* in RAW264.7 cells. Pre-treated macrophages were infected for 0 and 30 min and the bacterial internalization efficiency of *Brucella* was determined in (A) CXCR4-neutralizing antibody-treated RAW 264.7 cells (1 μg/ml), (B) CXCR4 siRNA-treated RAW 264.7 cells (10 μM), (C) AMD3100-treated RAW 264.7 cells (25 μg/ml). (D) Intracellular signaling was then assessed using phospho-specific and pan-antibodies against ERK1/2, JNK and p38α in CXCR4 knockdown cells. * $P < 0.05$, compared with *Brucella*-infected group.

2.13. Statistical analysis

Experiments were repeated at least two times and experimental groups typically consisted of five mice. The figures show the data from a representative experiment. The statistical differences between the values for different groups were evaluated by (GraphPad InStat) and $P < 0.05$ was taken as the level of significance.

3. Results

3.1. Effect of CXCR4 in the uptake of Brucella into RAW264.7 cells

RAW 264.7 cells pre-treated with CXCR4-neutralizing antibody for 30 min and then infected with *B. abortus* at indicated times showed that the uptake of *Brucella* was significantly reduced at 30 min ($P < 0.05$) post-infection (p.i.) as compared with the control (Fig. 1A). Consequently, the role of CXCR4 in RAW 264.7 cells was evaluated using small interfering RNA (siRNA). Efficiency of transfection was confirmed by quantitative RT-PCR (Fig. 1B) and cytotoxicity effect was evaluated using MTT assay (data not shown). The CXCR4 knockdown cells showed similar reduction of bacterial uptake at 30 min ($P < 0.01$) p.i. (Fig. 1B). Since blocking of CXCR4 in RAW 264.7 cells using siRNA reduced bacterial uptake, we further evaluated treatment of cells with CXCR4-specific antagonist AMD3100 as previously reported (Rosenkilde et al., 2003; Seemann and Lupp, 2016; Mishra et al., 2016). Incubation of cells for 30 min prior to infection with the antagonist also reduced bacterial uptake at 30 ($P < 0.01$) min p.i. (Fig. 1C). Since interference of CXCR4 led to inhibition of bacterial invasion in macrophages, we checked the involvement of mitogen-activated kinase (MAPK) in macrophages which plays an important role in the phagocytosis of microbes (Reyes et al., 2017b). The results revealed that *Brucella* infection in CXCR4-lacking cells at 30 min p.i. downregulated the phosphorylation level of ERK 1/2 ($P < 0.01$) while modest decrease was observed in JNK and p38α as compared with *B. abortus*-infected cells (Fig. 1D). Overall, our results indicate that CXCR4 blockade participates in the control of *Brucella* invasion into macrophages possibly via MAPKs pathway.

3.2. Effect of CXCR4 on the intracellular survival of Brucella and NO production in macrophages

Brucella survival within RAW 264.7 cells was not affected when the cells were incubated with CXCR4-neutralizing antibody (Figs. 2A) or CXCR4 siRNA for 2, 24 and 48 h (Figs. 2B) while intracellular growth was attenuated only at 2 ($P < 0.05$) h for the cells incubated with AMD3100 (Fig. 2C) indicating that CXCR4 blockade does not play an essential role in the control of *Brucella* survival within macrophages. On the other hand, *Brucella* persistence has been associated with effector molecules including nitric oxide (NO) reported to be involved in the killing of a number of intracellular pathogens (Grillo et al., 2012; Hop et al., 2018). In this study, a significant reduction in nitrite production was observed in *Brucella*-infected cells treated with CXCR4 neutralizing antibody ($P < 0.0001$) or AMD3100 ($P < 0.001$) at 24 h p.i. (Figs. 2D, E). Reduced nitrite production was also observed in CXCR4 knockdown cells ($P < 0.05$) at 48 h p.i. (Fig. 2F). These results suggest that CXCR4 could mediate NO molecules during *Brucella* infection and its blockade could render protection against further cell damage during bacterial infection.

3.3. Effect of CXCR4 antagonist in murine brucellosis

During the entire period of experiment, all treatment groups were monitored and no clinical symptoms were observed. In the non-infected groups, we checked the total body weight (Fig. 3A) and serum ALT concentrations (Fig. 3B) and the results showed no differences between treated and AMD3100-treated groups indicating that AMD3100 treatment did not negatively affect the weight and liver function of the animals. Furthermore, the total weight of the spleens in either of the group was not significantly different (Fig. 3C). On the other hand, in the *Brucella*-infected groups, the total weight of the spleens collected from

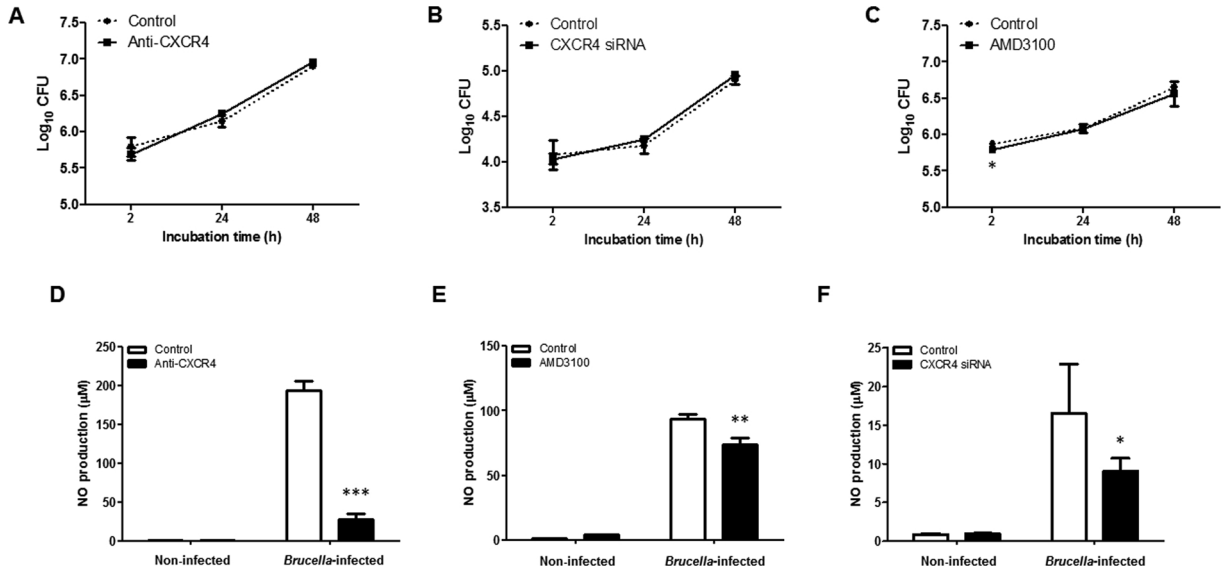

Fig. 2. Effects of CXCR4 blockade on the intracellular survival of *B. abortus* and NO production in RAW264.7 cells. Macrophages were incubated with (A) CXCR4-neutralizing antibody (1 μg/ml), (B) CXCR4 siRNA and (C) AMD3100 (25 μg/ml). Nitrite production was measured in cells incubated with (D) CXCR4-neutralizing antibody (24 h p.i.), (E) AMD3100 (24 h p.i.) or (F) CXCR4 siRNA (48 h p.i.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with *Brucella*-infected group.

AMD3100-treated mice was significantly higher (0.224 ± 0.018 g) ($P < 0.05$) than the total weight of the *Brucella*-infected group (0.198 ± 0.011 g) (Fig. 4A). However, a significant reduced bacterial burden was recovered from the spleens of AMD3100-treated mice (5.29 ± 0.13) ($P < 0.05$) as compared with *Brucella*-infected group (6.00 ± 0.09) (Fig. 4B). We then evaluated the direct effect of AMD3100 against *Brucella* growth at 24 h incubation and found that the antagonist significantly inhibited the growth of the bacteria ($P < 0.001$) indicating that AMD3100 has a bactericidal effect against *B. abortus* (Fig. 4C). The serum samples from these mice were evaluated to determine the effects of AMD3100 treatment on cytokine production. In comparison to non-infected groups, AMD3100-treated mice displayed elevated level of TNF ($P < 0.05$) and reduced level of IL-10 ($P < 0.05$) at 3 d p.i. (Fig. 4D). In the *Brucella*-infected groups at 3 d p.i., AMD3100-treated group displayed reduced level of IL-1β ($P < 0.05$) and increased level of IL-12 ($P < 0.001$) (Fig. 4D) as compared to control. We observed that at 14 days p.i., levels of IL-1β ($P < 0.0001$) and IL-6 ($P < 0.01$) declined while IL-12 ($P < 0.01$),

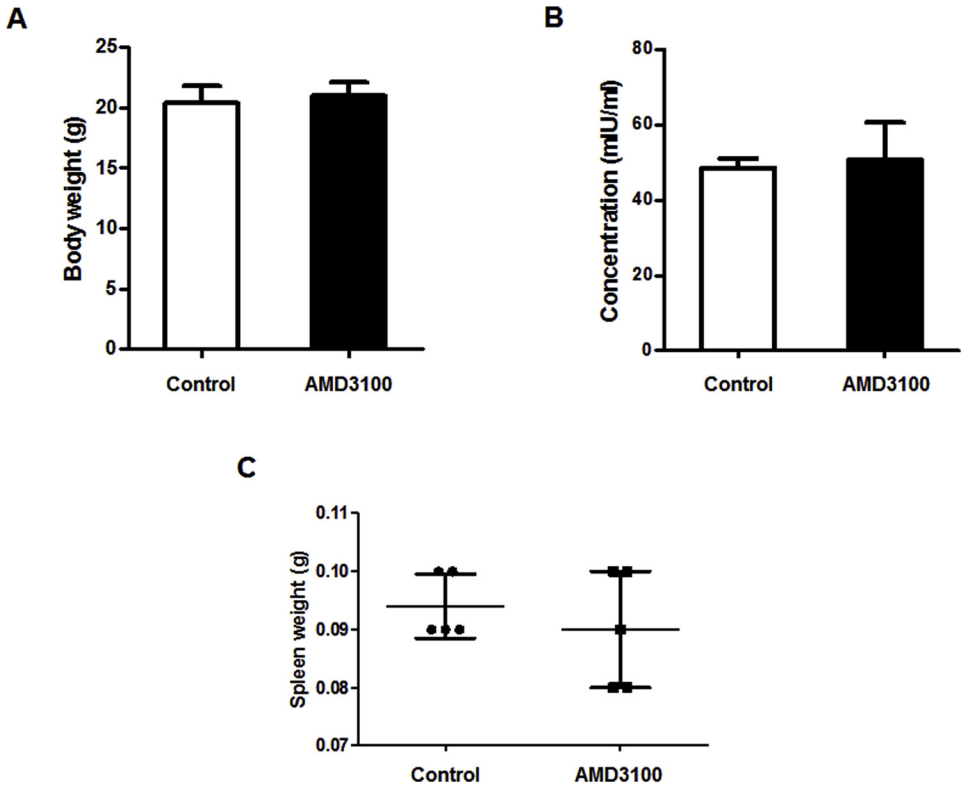

Fig. 3. Effects of CXCR4 antagonist AMD3100 in BALB/c mice. Mice were pretreated intraperitoneally with AMD3100 or PBS at different time points. At the end of the experiment, (A) total body weight, (B) serum ALT concentrations and (C) total weight of spleens were determined in non-infected groups.

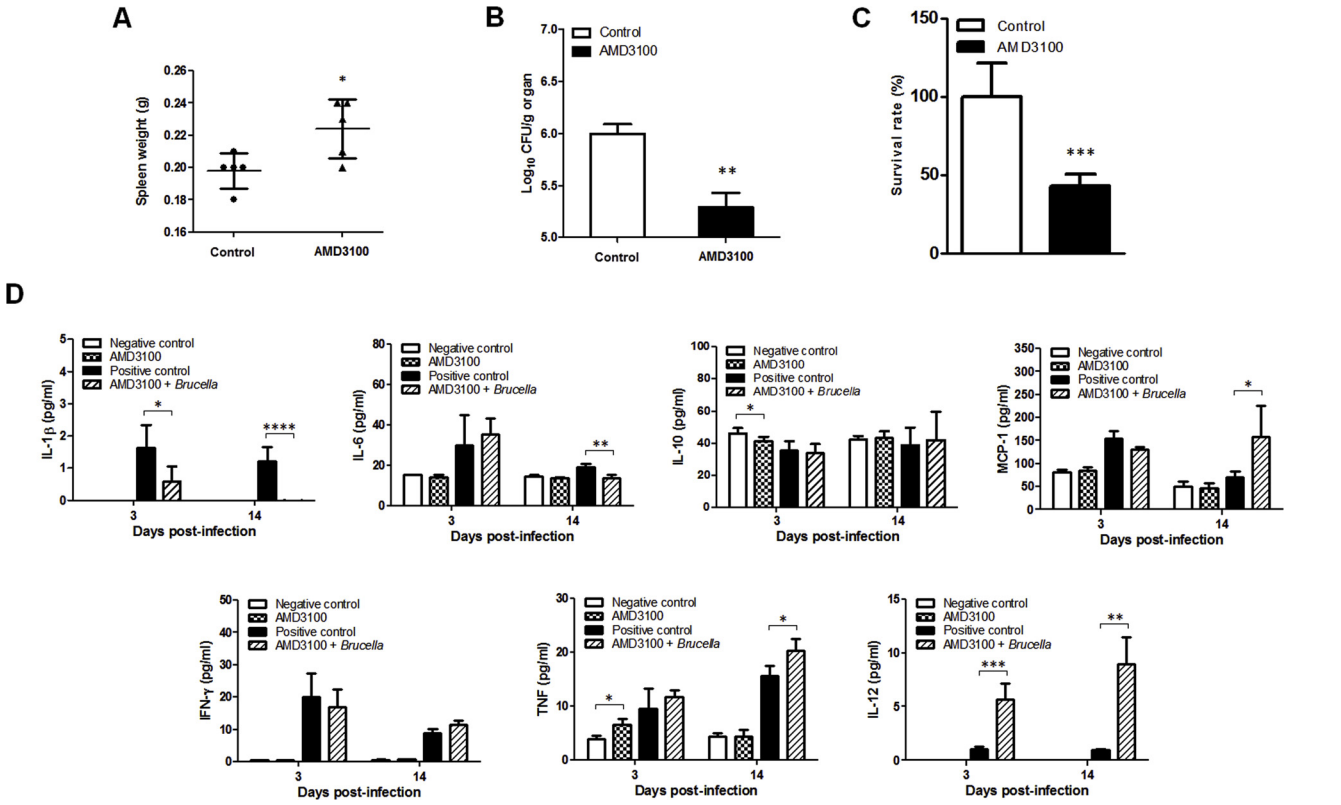

Fig. 4. Effects of CXCR4 antagonist AMD3100 on bacterial splenic proliferation and immune response to *B. abortus* in mice. At 14 d p.i., mice were sacrificed and (A) the total weight of spleens in AMD3100 or PBS-treated group was evaluated. (B) The number of bacterial CFUs in each spleen was determined. (C) Direct effect of AMD3100 (25 μg/ml) on the growth of *B. abortus* was also assessed at 24 h. (D) Cytokine levels from serum samples were analyzed using CBA analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, compared with control group.

MCP-1 ($P < 0.05$) and TNF ($P < 0.05$) increased in AMD3100-treated mice as compared to control (Fig. 4D).

4. Discussion

Brucella is a bacterial pathogen that causes brucellosis, a worldwide zoonosis of profound importance due to elusion from systematic attempts of eradication. For more than a century, there is no up to date approved human vaccine, and the recommended treatment requires prolonged administration of antibiotics that often fails to completely clear the infection - all contributing to the classification of the pathogen as a potential bioterror agent and economic threat (Gomez et al., 2013; Pandey et al., 2018). Host-pathogen interaction is of great significance as it dictates the course of infection and as a successful agent *Brucella* requires four steps including adherence, invasion, establishment and dissemination within the host (Ko and Splitter, 2003). In response to initial infection, host innate immune system represents an efficient first line of defense against *Brucella* and macrophages are key elements against this intracellular pathogen, however *Brucella* can elude initial immune recognition resulting in a mild pro-inflammatory response that leads to bacterial persistence (Jiang et al., 2018). CXCR4 is expressed on a wide variety of leukocytes and on cells outside the immune system such as in the central nervous system, and appears to be exploited by pathogens as a mechanism of immune evasion and its inhibition has been suggested to be an important therapeutic strategy in many circumstances such as cancer, HIV-1 pathogenesis, human periodontitis and associated systemic diseases, inflammation, stem cell migration and autoimmune diseases (Verani et al., 2002; Rosenkilde et al., 2004; Hajishengallis et al., 2008; Mishra et al., 2016). In our study, we have presented data on evasion strategy against *B. abortus* in macrophages via CXCR4 blockade using neutralizing antibody, siRNA and specific antagonist suggesting the important role of CXCR4 in favor of *Brucella* invasion into host cells. AMD3100, a specific CXCR4 antagonist, is known to essentially bind to three acidic anchor-point residues at the main ligand-binding pocket of CXCR4 (Rosenkilde et al., 2004). Our findings require further investigations particularly on direct interaction of the pathogen with the receptor for its successful infection as well as contribution of SDF-1 during brucellosis. Several studies verified that CXCR4 signaling also contributed to phosphorylation of MAPKs (Hu et al., 2014; Isaacson et al., 2017; Dinkel et al., 2018). Similarly, we found that CXCR4 blockade using siRNA attenuated ERK1/2 phosphorylation during *B. abortus* infection indicating that inhibition of bacterial internalization is possibly mediated via ERK pathway. In contrast, survival of *Brucella* within macrophages during the course of 24 until 48 h was not affected when CXCR4 was inhibited suggesting that participation of CXCR4 is limited at the bacterial phagocytic pathway. NO, on the other hand, is known as a toxic molecule of the immune system which contributes to the control of pathogens and tumors, modulation of cytokine response of lymphocytes, regulation of immune cell apoptosis and immunodeviating effects (Bogdon et al., 2000). In a study done by Nagashima et al. (2017b), NO metabolites after *P. gingivalis* inoculation in the gingival tissue were reduced by gingival CXCR4 neutralization. Similarly, CXCR4 blockade in the present study resulted in reduced NO production in macrophages during *B. abortus* infection. CXCR4 is activated by a single chemokine ligand - stromal cell-derived factor (SDF-1α or CXCL12) (Rosenkilde et al., 2004) and it was reported that production of NO was observed from SDF-1α-induced activation of JNK3 in endothelial cells (Pi et al., 2009).

Cytokines are important in the host defense system as they play a critical role in protection against microbial infection but are also involved in the pathogenesis and development of symptoms (Imanishi, 2000). Liver and spleens are the most conspicuously infected organs in mice after *Brucella* infection but spleen has shown to have higher number of *Brucella* CFU per gram of organ at two weeks post-infection

(Grillo et al., 2012). Serum cytokine level reflects cytokine production and secretion from splenic cells (Athanassakis and Iconomidou, 1996). CXCR4 transactivation has been reported to induce robust cytokine expression particularly pro-inflammatory cytokines elucidating the role of CXCR4 in inflammation (Dinkel et al., 2018; Hu et al., 2014). Here, we have also demonstrated that AMD3100 treatment in mice led to different expression of several cytokines from serum samples in response to *Brucella* infection. Seemann and Lupp (2016) demonstrated that CXCR4 blockade using AMD3100 in mice showed enhanced serum levels of TNF-α and IFN-γ in endotoxemia. *P. gingivalis*-stimulated human monocytes pretreated with anti-CXCR4 monoclonal antibody strikingly down-regulated IL-10 secretion (Hajishengallis et al., 2008). Anti-inflammatory IL-10 functions to control excessive inflammatory response preventing further tissue damage (Spera et al., 2014). However, IL-10 is also known to promote chronic infections such as *B. abortus* persistence and as a key inhibitor of pro-inflammatory molecules such as IL-1β, TNF, IL-6, GM-CSF, MIP-1α, and NO in LPS-activated macrophages (Spera et al., 2014). Induction of pro-inflammatory cytokines is directly related to protective immunity against *B. abortus* and is associated with the strategy of the pathogen to establish a chronic infection to host (Spera et al., 2014). In our study, AMD3100 treatment showed reduced IL-1β and IL-6 while augmented TNF, IL-12 and MCP-1 production during murine brucellosis. Overproduction of IL-1β is implicated in the painful and inflammatory processes suggesting that its blockade could be considered as a therapeutic opportunity, and through complex signaling cascades can lead to release of IL-6 (Ren and Torres, 2009). IL-6, on the other hand, is sometimes viewed as anti-inflammatory cytokine that may cause disease and injury in prolonged duration (Cahill and Rogers, 2008). Furthermore, CXCR4 blockade using antibody, siRNA and AMD3100 in oral squamous cell carcinoma inhibited SDF-1α-induced increase IL-6 production that promotes osteoclastogenesis (Tang et al., 2008). TNF is shown to be involved in the control of *Brucella* inside macrophages as well as in murine brucellosis (Macedo et al., 2008). Administration of recombinant IL-12 in MyD88 knockout mice enhanced resistance to *Brucella* indicating that lack of production of this cytokine is partially responsible for enhanced susceptibility to brucellosis (Macedo et al., 2008). MCP-1 has been associated in the recruitment of inflammatory cells at the site of infection as well as in host immunity to *B. abortus* infection in humans and mice (Scian et al., 2011).

Seemann and Lupp (2016) reported that co-administration of AMD3100 and LPS resulted in splenomegaly in C57BL/6 N mice suggesting that CXCR4 modulation is of critical importance in endotoxemia. Similarly, splenomegaly was also observed in AMD3100-treated mice in the present study although we used a much lower and safe dose as confirmed in the in vitro analysis. Furthermore, AMD3100 treatment was also observed to reduce the growth of *Brucella* in vitro which could be attributed to reduced number of bacteria that survived within macrophages as well as attenuated splenic bacterial CFUs. However, further studies are needed to confirm the direct effect of AMD3100 against *Brucella* microorganism during infection both in vitro and in vivo systems. Overall, manipulation of CXCR4 can be further explored in finding new therapeutics or counterstrategy against brucellosis and possibly other intracellular pathogens.

Author contributions

AWBR carried out experiments, analyzed the data and wrote the manuscript. LTA, TXNH and SHV contributed in the experiments and revision of the manuscript. CKK, WM, HJL and SK developed the concepts, designed the experiments and provided the reagents.

Declaration of Competing Interest

The authors declared no potential conflicts of interest.

Acknowledgement

This research was supported by a fund (Project Code No. Z-1543061-2019-20-01) from Research of Animal and Plant Quarantine Agency, South Korea.

References

Athanassakis, I., Iconomidou, B., 1996. Cytokie production in the serum and spleen of mice from day 6 to 14 of gestation: cytokine/placenta/spleen/serum. Dev. Immunol. 4, 247–255.

Bogdon, C., Rollinghoff, M., Diefenbach, A., 2000. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr. Opin. Immunol. 12, 64–76.

Busillo, J.M., Benovic, J.L., 2007. Regulation of CXCR4 signaling. Biochim. Biophys. Acta 1768, 952–963.

Cahill, C.M., Rogers, J.T., 2008. Interleukin (IL) 1β induction of IL-6 is mediated by a novel phosphatidylinositol 3-kinase-dependent AKT/IκB kinase α pathway targeting activator protein-1. J. Biol. Chem. 283, 25900–25912.

Cha, S.B., Rayamajhi, N., Lee, W.J., Shin, M.K., Jung, M.H., Shin, S.W., Kim, J.W., Yoo, H.S., 2012. Generation and envelope protein analysis of internalization defective *Brucella abortus* mutants in professional phagocytes, RAW 264.7. FEMS Immunol. Med. Microbiol. 64, 244–254.

Dinkel, B.A., Kremer, K.N., Rollins, M.R., Medlyn, M.J., Hedin, K.E., 2018. GRK2 mediates TCR-induced transactivaton of CXCR4 and TCR-CXCR4 complex formation which drives PI3Kγ/PREX1 signaling and T cell cytokine secretion. J. Biol. Chem. 293, 14022–14039.

Franc, K.A., Krecek, R.C., Hasler, B.N., 2018. Arenas-Gamboa, A.M. Brucellosis remains a neglected disease in the developing world: a call for interdisciplinary action. BMC Public Health 18, 125.

Gomez, G., Adams, L.G., Rice-Ficht, A., Ficht, T.A., 2013. Host-*Brucella* interactions and the *Brucella* genome as tools for subunit antigen discovery and immunization against brucellosis. Front. Cell. Infect. Microbiol. 3, 17.

Grillo, M.J., Blasco, J.M., Gorvel, J.P., Moriyon, I., Moreno, E., 2012. What have we learned from brucellosis in mouse model? Vet. Res. 43, 29.

Hajishengallis, G., Wang, M., Liang, S., Triantafilou, M., Triantafilou, K., 2008. Pathogen induction of CXCR4/TLR2 cross-talk impairs host defense function. Proc. Natl. Acad. Sci. U. S. A. 105, 13532–13537.

Hop, H.T., Arayan, L.T., Huy, T.X.N., Reyes, A.W.B., Baek, E.J., Min, W., Lee, H.J., Rhee, M.H., Watanabe, K., Chang, H.H., Suk, Kim., 2018. Lipocalin 2 (Lcn2) interferes with iron uptake by *Brucella abortus* and dampens immunoregulation during infection of RAW 264.7 macrophages. Cell. Microbiol. 20, e12813.

Hu, X.M., Liu, Y.N., Zhang, H.L., Cao, S.B., Zhang, T., Chen, L.P., Shen, W., 2014. CXCL12/CXCR4 chemokine signaling in spinal glia induces pain hypersensitivity through MAPKs-mediated neuroinflammation in bone cancer rats. J. Neurochem. 452–463.

Imanishi, J., 2000. Expression of cytokines in bacterial and viral infections and their biochemical aspects. J. Biochem. 127, 525–530.

Isaacson, B., Hadad, T., Glasner, A., Gur, C., Granot, Z., Bachrach, G., Mandelboim, O., 2017. Stromal cell-derived factor 1 mediates immune cell attraction upon urinary tract infection. Cell Rep. 20, 40–47.

Jiang, H., Dong, H., Peng, X., Feng, Y., Zhu, L., Niu, K., Peng, Y., Fan, H., Ding, J., 2018. Transcriptome analysis of gene expression profiling of infected macrophages between *Brucella suis* 1330 and live attenuated vaccine strain S2 displays mechanistic implication for regulation of virulence. Microb. Pathog. 119, 241–247.

Jung, M., Shim, S., Im, Y.B., Park, W.B., Yoo, H.S., 2018. Global gene-expression profiles of intracellular survival of the BruAb2_1031 gene mutated *Brucella abortus* in professional phagocytes, RAW 264.7 cells. BMC Microbiol. 18, 82.

Khan, M.Z., Zahoor, M., 2018. An overview of brucellosis in cattle and humans, and its serological and molecular diagnosis in control strategies. Trop. Med. Infect. Dis. 3, 65.

Kishore, S.P., Bungum, M.K., Platt, J.L., Brunn, G.J., 2005. Selective suppression of Toll-like receptor 4 activation by chemokine receptor 4. FEBS Lett. 579, 699–704.

Ko, J., Splitter, G.A., 2003. Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. Clin. Microbiol. Rev. 16, 65–78.

Macedo, G.C., Magnani, D.M., Carvalho, N.B., Bruna-Romero, O., Gazzinelli, R.T., Oliveira, S.C., 2008. Central role of MyD88-dependent dendritic cell maturation and proinflammatory cytokine production to control *Brucella abortus* infection. J. Immunol. 180, 1080–1087.

Mishra, R.K., Shum, A.K., Platanias, L.C., Miller, R.J., Schiltz, G.E., 2016. Discovery and characterization of novel small-molecule CXCR4 receptor agonists and antagonists. Sci. Rep. 6, 30155.

Nagashima, H., Shinoda, M., Honda, K., Kamio, N., Watanabe, M., Suzuki, T., Sugano, N., Sato, S., Iwata, K., 2017a. CXCR4 signaling in macrophages contributes to periodontal mechanical hypersensitivity in *Porphyromonas gingivalis*-induced periodontitis in mice. Mol. Pain 13, 1–8.

Nagashima, H., Shinoda, M., Honda, K., Kamio, N., Watanabe, M., Suzuki, T., Sugano, N., Sato, S., Iwata, K., 2017b. CXCR4 signaling in macrophages contributes to periodontal mechanical hypersensitivity in *Porphyromonas gingivalis*-induced periodontitis in mice. Mol. Pain 13, 1–8.

Pandey, A., Lin, F., Cabello, A.L., da Costa, L.F., Feng, X., Feng, H.Q., Zhang, M.Z., Iwawaki, T., Rice-Ficht, A., Ficht, T.A., de Figueiredo, P., Qin, Q.M., 2018. Activation of host IRE1α-dependent signaling axis contributes the intracellular parasitism of

Brucella melitensis. Front. Cell. Infect. Microbiol. 8, 103.
Pi, X., Wu, Y., Ferguson III, J.E., Portbury, A.L., Patterson, C., 2009. SDF-1α stimulates JNK3 activity via eNOS-dependent nitrosylation of MKP7 to enhance endothelial migration. Proc. Natl. Acad. Sci. U. S. A. 106, 5675–5680.
Ren, K., Torres, R., 2009. Role of interleukin-1β during pain and inflammation. Brain Res. Rev. 60, 57–64.
Reyes, A.W.B., Hop, H.T., Arayan, L.T., Huy, T.X.N., Min, W., Lee, H.J., Chang, H.H., Kim, S., 2017a. Nocodazole treatment interrupted *Brucella abortus* invasion in RAW 264.7 cells, and successfully attenuated splenic proliferation with enhanced inflammatory response in mice. Microb. Pathog. 103, 87–93.
Reyes, A.W.B., Hop, H.T., Arayan, L.T., Huy, T.X.N., Park, S.J., Kim, K.D., Min, W., Lee, H.J., Rhee, M.H., Kwak, Y.S., Kim, S., 2017b. The host immune enhancing agent Korean red ginseng oil successfully attenuates *Brucella abortus* infection in a murine model. J. Ethnopharmacol. 198, 5–14.
Rosenkilde, M.M., Gerlach, L.O., Jakobsen, J.S., Skerlj, R.T., Bridger, G.J., Schwartz, T.W., 2004. Molecular mechanism of AMD3100 antagonism in the CXCR4 receptor: transfer of binding site to the CXCR3 receptor. J. Biol. Chem. 279, 3033–3041.
Scian, R., Barrionuevo, P., Giambartolomei, G.H., De Simone, E.A., Vanzulli, S.I., Fossati, C.A., Baldi, P.C., Delpino, M.V., 2011. Potential role of fibroblast-like synoviocytes in joint damage induced by *Brucella abortus* infection through production and induction of matrix metalloproteinases. Infect. Immun. 79, 3619–3632.
Seemann, S., Lupp, A., 2016. Administraton of AMD3100 in endotoxemia is associated with pro-inflammatory, pro-oxidative, and pro-apoptotic effects in vivo. J. Biomed. Sci. 23, 68.
Spera, J.M., Comerci, D.J., Ugalde, J.E., 2014. *Brucella* alters the immune response in a *prpA*-dependent manner. Microb. Pathog. 67-68, 8–13.
Tang, C.H., Chuang, J.Y., Fong, Y.C., Maa, M.C., Way, T.D., Hung, C.H., 2008. Bone-derived SDF-1α stimulates IL-6 release via CXCR4, ERK and NF-κB pathways and promotes osteoclastogenesis in human oral cancer cells. Carcinogenesis 29, 1483–1492.
Tissir, F., Wang, C.E., Goffinet, A.M., 2004. Expression of the chemokine receptor Cxcr4 mRNA during mouse brain development. Dev. Brain Res. 149, 63–71.
Triantafilou, M., Lepper, P.M., Briault, C.D., Ahmed, M.A.E., Dmochowski, J.M., Schumann, C., Triantafilou, K., 2008. Chemokine receptor 4 (CXCR4) is part of the lipopolysaccharide “sensing apparatus”. Eur. J. Immunol. 192–203.
Verani, A., Sironi, F., Siccardi, A.G., Lusso, P., Vercelli, D., 2002. Inhibition of CXCR4-trophic HIV-1 infection by lipopolysaccharide: evidence of different mechanisms in macrophages and T lymphocytes. J. Immunol. 168, 6388–6395.