Comparative Analysis of Antimicrobial Peptides Gene Expression in Susceptible/Resistant Mice Macrophages to Leishmania major Infection

Hamid Daneshvar (1)
Iraj Sharifi (2,3)
Alireza Kyhani (3)
Amir Tavakoli Kareshk (2)
Arash Asadi (2)

(1) Department of Immunology, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran
(2) Department of Parasitology and Mycology, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran
(3) Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran

Corresponding author:
Arash Asadi
Department of Parasitology and Mycology,
School of Medicine, Kerman University of Medical Sciences,
Kerman, Iran
Email: asadiarash209@yahoo.com

Abstract

Introduction and Objective: BALB/c and C57BL/6 mouse strains represent immunologically different responses to *Leishmania* major infection. Antimicrobial peptides (AMPs) for example, cathelicidins and defensins, are unique compounds of innate immunity system with multifunctional effects against invasive pathogens. Nevertheless, they have been less studied in parasitic fields. The aim of the present study was to evaluate the role of AMPs in susceptibility or resistance to *L.major* infection.

Methodology: Macrophages derived from peritoneal cavity of BALB/c and C57BL/6 mouse strains were exposed to the stationary phase of *L. major* promastigotes for 3 hours, 24 hours and 7 days. Cell sediments and supernatants from infected (test) and uninfected groups (control) at 3 hours, 24 hours and on 7 days were used for the assessment of infection severity, gene expression of various mouse beta defensins (mBD), Cathelin-related antimicrobial peptide (CRAMP), interleukin (IL)-10, IL-12 and protein assay under standard methods, respectively.

Findings: Based on cytokine profiles evaluated in BALB/c (↑IL-10, ↓IL-12) and C57BL/6 derived macrophages (↓IL-10, ↑IL-12), the immunity system was stimulated differently during infection. The inter assay analysis revealed that the test group of BALB/c derived macrophages significantly expressed an up-regulation of CRAMP, mBD1 genes and their related proteins, when they are challenged with *L. major* parasites. Nevertheless, they also showed infection severity more than those in other strains.

Conclusion: Due to higher expression and release of AMPs by BALB/c derived macrophages, the *L. major* infection ultimately occurs in BALB/c mice. On the other hand, the release of AMPs is important, but cannot create an absolute protection against leishmania infection.

Key words: Antimicrobial Peptides, Cathelin-Related Antimicrobial Peptide, Murine b-Defensin, *Leishmania major*, Cytokine

Please cite this article as: Asadi, A, et al. Comparative Analysis of Antimicrobial Peptides Gene Expression in Susceptible/Resistant Mice Macrophages to Leishmania major Infection . *World Family Medicine*. 2017; 15(9):18-24. DOI 10.5742/MEWFM.2017.93096

Introduction

Leishmaniasis is an arthropod-borne disease created by intracellular protozoan parasites of the *Leishmania* genus (Vega-López, 2012). It is a very important health problem of the recent century in 98 countries and territories (Alvar et al., 2012). The endemic areas of human infections are present mainly in tropics, subtropics, southern Europe and western Asia (Ashford, 1997, Desjeux, 1996). Depending on species and host immunity, several complications such as cutaneous (CL), muccocutaeous (MCL) and visceral leishmaniasis (VL) have been recognized (Herwaldt, 1999). *L. major* is an ethological agent of CL in different countries such as Iran (Le Blancq et al., 1986, Azizi et al., 2016). Infection is caused, when a female sand-fly inoculates the metacyclic phase of *L. major* promastigotes into the dermis of a suitable vertebrate host (Dostálová and Volf, 2012). The parasites are engulfed by macrophages for further development (Handman and Bullen, 2002). More interestingly, macrophages involve in both development and killing of parasites. Previous studies have shown that susceptible (BALB/c) and resistant (C57BL/6) mouse strains represent different immune responses to *L. major* infection (Hejazi et al., 2012, Lazarski et al., 2013, Park et al., 2000). Based on the important role of macrophages, it is necessary to know if new mechanisms such as antimicrobial peptides (AMPs) are employed by them following *L. major* infection. Historically, the first AMPs was isolated from a soil Bacillus strain and named gramicidin (Dubos, 1939). More than 5,000 AMPs have been identified so far (Zhao et al., 2013). Cathilicidins and defensins are two main groups of AMPs (Ganz, 2003, Lehrer and Ganz, 2002a). Cathelin-related antimicrobial peptide (CRAMP) is the only cathelicidin found in mouse strains and expressed by different kinds of cells or tissues, while a variety of mouse beta defensins (mBD) have been identified (Dorschner et al., 2003, Nizet and Gallo, 2003, Bardan et al., 2004). They kill or inhibit invasion pathogens through direct effects or modulation of inflammatory responses (Deng et al., 2016, Hemshekhar et al., 2016, Chromek et al., 2012, Kovach et al., 2012). Despite being remarkable cases of CL, little study is found about AMPs role in Leishmania infections. The present study aimed to show whether AMPs can affect susceptibility or resistance to *L. major* infection.

Materials and Methods

Ethics approval and consent to participate: To work on animals, we obtained permission number ir.kmu.rec.1394.208 from the ethical board of Kerman University of Medical Science (Kerman, Iran).

Parasite: *L. major* (strain MRHO/IR/75/ER, Iranian type collection) was purchased from Razi Institute (Karj, Iran) and cultured in 50 ml flask containing RPMI 1640 enriched with 10% heated-inactivated fetal bovine serum (HFBS) and 1% penicillin/streptomycin (pen/strep) antibiotics.

Macrophages Isolation: Macrophages were isolated from BALB/c (n=5) and C57BL/6 (n=5) mouse strains from peritoneal cavity like the previous study (Ray and Dittel, 2010), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% HFBS and 1% pen/strep antibiotics. The cells were incubated at 37oC in 5% CO2 in humid conditions. For the experiments, the macrophages derived from each strain were separately placed in two sub groups: non-infected (control) and challenged by *L. major* (test).

Co-incubation of Macrophages with parasite: Macrophage (10^6 /well) from both strains were separately transferred into 24-wells cell culture plates. Each cell culture plate was designated for one defined time and selected group (5 well for test groups and 5 well for controls). The cells were incubated at 37°C for 6 hours and non adherent cells removed. The stationary phase of *L. major* promastigotes (10:1) was added only to test groups and incubated at 37°C for 3 hours. The free promastigotes were removed and the cells were incubated at 37°C for an additional 24 hours and 7 days.

Microscopic observation: To measure parasite burden, the test groups were stained using routine Giemsa staining method 3 hours post infection according to a previous study (Faber et al., 2003). Parasite burden (number of parasites per macrophage) and infection rate (% infected macrophages) were obtained by counting the intracellular amastigotes using a light microscope (Nikon, Japan).

Quantitative Real-Time PCR: For analysis of cytokines and AMPs gene expression, whole macrophages from test and control groups were separately harvested at 24 hours post infection with parasites. Total RNA was extracted using RNA Purification kit (Jena Bioscience, Germany) and quantified by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Three mg was transcribed to complementary DNA (cDNA) using AccuPower®RT PreMix random hexaprimer (Bioneer, Korea). Briefly, 3 µg of RNA was adjusted in 20 µl DEPCI-DW and totally added to each lyophilized tube. Thermal profile was performed as following: 12 cycles (20ºC for 30 seconds, 42ºC for 4 minutes, 55ºC for 30 seconds) and 95ºC for 5 minutes. Quantitative Real-time PCR was utilized using a Rotor GENE Q (Qiagen, Germany). RPII was used to amplify house-keeping cDNA. Other primers were applied to amplify desirable amount of cDNA (Table 1). Briefly, The 15µl of each reaction mixture (1ml cDNA, 7 ml SYBR Green, 5ml DW, 1mL primer forward 2.5 Pmol, 1mL primer reverse 2.5 Pmol) was prepared using SYBR Premix EX Taq2 Master Mix (Takara, Japan). Thermal profile was performed as following: 95°C for 1 minute, 40 cycles (94°C for 15 seconds, 58°C for 30 seconds, 72°C for 20 seconds).

ELISA for protein assay: Macrophages were exposed with the stationary phase of *L. major* promastigotes and kept for 7 days. Due the fact that CRAMP is secreted into cell culture media by macrophages, supernatants were collected for CRAMP assessment by enzyme-linked immunosorbent assay (ELISA). CRAMP assay was accessed using direct ELISA. In this method, the concentration of CRAMP is equal with the absorbance of optical density (OD), and

mean of the final OD was calculated as final results. Briefly, a 96-well plate was coated with 5 µg of each supernatant in 50 µl of 0.1M carbonate buffer PH 9.6 and incubated at 4°C FOR 18h. After 3 washes with 300µl of PBS, pH 7.2, 0.1% Tween-20, the plate was then blocked with 100 µl of blocking buffer (PBS, FBS 10%) and incubated at 37°C for 1 hour. Following 3 washes, 100 µl of 1:200 (in PBS, Ph 7.2, 0.1% Tween-20) of horse radish peroxidase conjugated CRAMP antibody (Santacruz, California) was added and incubated for 1 hour at 37º C and washed 3 times at the end of incubation. The plate was incubated with 100 µl of substrate solution for 30 minutes. In the final step, 50µl of stop solution was added and optical density (OD_{492}) detected using ELx800 micro plate reader (BioTek, USA).

Findings

Parasite burden: Initially, we assessed infectivity rate and parasite burden of test groups 3 hours post co-incubation. C57BL/6 derived macrophages had a significant reduction of infection rate (24.5±0.31) as compared to (45±0.73) for BALB/C derived macrophages (Figure 1. A). In the next step, we characterized the parasite burden by counting the number of intracellular amastigotes per macrophage. We saw a significant reduction of parasite burden (2.78±0.10 parasite/macrophage) for C57BL/6 derived macrophages as compared to (8.68±0.22) for other strain (Figure 1. B).

Antimicrobial peptides expression: Real-time PCR was applied to measure the mRNA of defined AMPs following L. major infection. The results were analyzed under ΔΔCT method. In BALB/c derived macrophages, the test groups expressed all aforementioned genes more than their controls except mBD2, but significantly up-regulation was documented only for CRAMP and mBD1 (Figure 2. A). Unlike the BALB/c macrophages, the test groups of C57BL/6 derived macrophages slightly expressed all mentioned genes except mBD6 more than their controls, but there wasn't observed any significant differences between them (Figure 2. B). Inter assay analysis showed that the test groups of BALB/c derived macrophages significantly expressed a high level of CRAMP (3.2507±0.0499) and mBD1 (3.0362±0.0701) compared to (0.9852±0.0267) and (1.2074±0.0418) in C57BL/6 derived macrophages, respectively (Figure 2. C - page 22).

Cytokines expression: Cytokines expression was assessed using real-time PCR method, and the findings analyzed under ΔΔCT method. In BALB/C derived macrophages, the test groups expressed a low level of IL-12 (0.96 ± 0.04) and a significant level of IL-10 (1.91± 0.02) in comparison to (1.005±0.0134) and (1.0016±0.0231) for their controls, respectively (Figure 3. A - page 22). Instead, the test groups of C57BL/6 derived macrophages showed significant expression levels of IL-12 (2.19± 0.05) and a low level of IL-10 (0.85 ± 0.01) versus (1.0036±0.0347) and (1.0021±0.0260) for their controls (Figure 3. B).

Protein assay: Based on AMPs genes expression, the test groups of BALB/c derived macrophages expressed the mRNA levels of CRAMP and mBD1 more than those in C57BL/6. Owing to higher expression of CRAMP compared to mBD1, protein assay was performed only for CRAMP 7 days post infection. Attention to data detected by ELISA method, the test groups of BALB/c derived macrophages released a high level of CRAMP (1.3447±0.010497) in comparison to their controls (0.4706±0.002537) and to (0.4862±0.0021) and (0.4803±0.0022) for the other strain according to the absorbance of optical density assessment (Figure 4 - page 23).

Discussion and Conclusion

Leishmaniasis is a public health problem in many countries (Stefaniak et al., 2002) and there are an estimated 700,000–1 million new cases each year (WHO. Fact sheet. April 2017). It takes a huge economic burden annually. *L. major* infection is an appropriate model to determine the necessity of immune responses to infection outcome. It has been proven that the increase of some immune effectors, such as IL-12, cause naive lymphocytes differentiate to Th2, which can produce IFNγ cytokine (Park et al., 2000). This cytokine plays a very important role in activating of macrophage cells. IFNγ-activated macrophages can destroy the intracellular parasites through a variety of well known mechanisms and induce resistance in C57BL/6 mouse strain (Assreuy et al., 1994). Instead the polarization of Th2 can ultimately predispose BALB/c mice to infection (Chatelain et al., 1992). Similarly to in vivo model, BALB/c and C57BL/6 derived macrophages represent different responses, when they are challenged with L. major parasites (Rabhi et al., 2013). It is possible for genetically different cell types to exhibit an unlike response to the same pathogen like *L. major*. There is a number of infections referred to as AMPs imbalance, in which they influence susceptibility or resistance to infections (Rivas-Santiago et al., 2009). Surprisingly, these peptides classified in the innate immunity of living organisms, can kill or inhibit pathogens in each category (Lehrer and Ganz, 2002a, Lehrer and Ganz, 2002b, Zasloff, 2002, Bardan et al., 2004, Cavalcante et al., 2017, Kao et al., 2016, Mello et al., 2017, Vieira-Girao et al., 2017). They can be used as new drugs or applied as vaccine and resistance to them is rare (Dabirian et al., 2013, Diamond, 2001, Hancock and Sahl, 2006). The present study aimed to show if these peptides can affect susceptibility or resistance to *L. major* infection. We designed an in vitro model for studying of AMPs in parasitic infection for the first time. The increased level of infection severity in BALB/c derived macrophages indicates that this type is more sensitive to *leishmania* infection (Fig1.A-B). According to Sunderkotter et al. (Sunderkötter et al., 1993), C57BL/6 derived macrophages infected by *L. major* parasites mature faster, which results in the reduction of their infection severity and susceptibility. The results derived from this survey revealed that BALB/c derived macrophages use AMPs especially CRAMP and mBD1more to reduce clinical symptoms (Figure 2. A). It seems that their susceptibility to infection is the immense criteria for the increase of AMPs. There are less in vitro

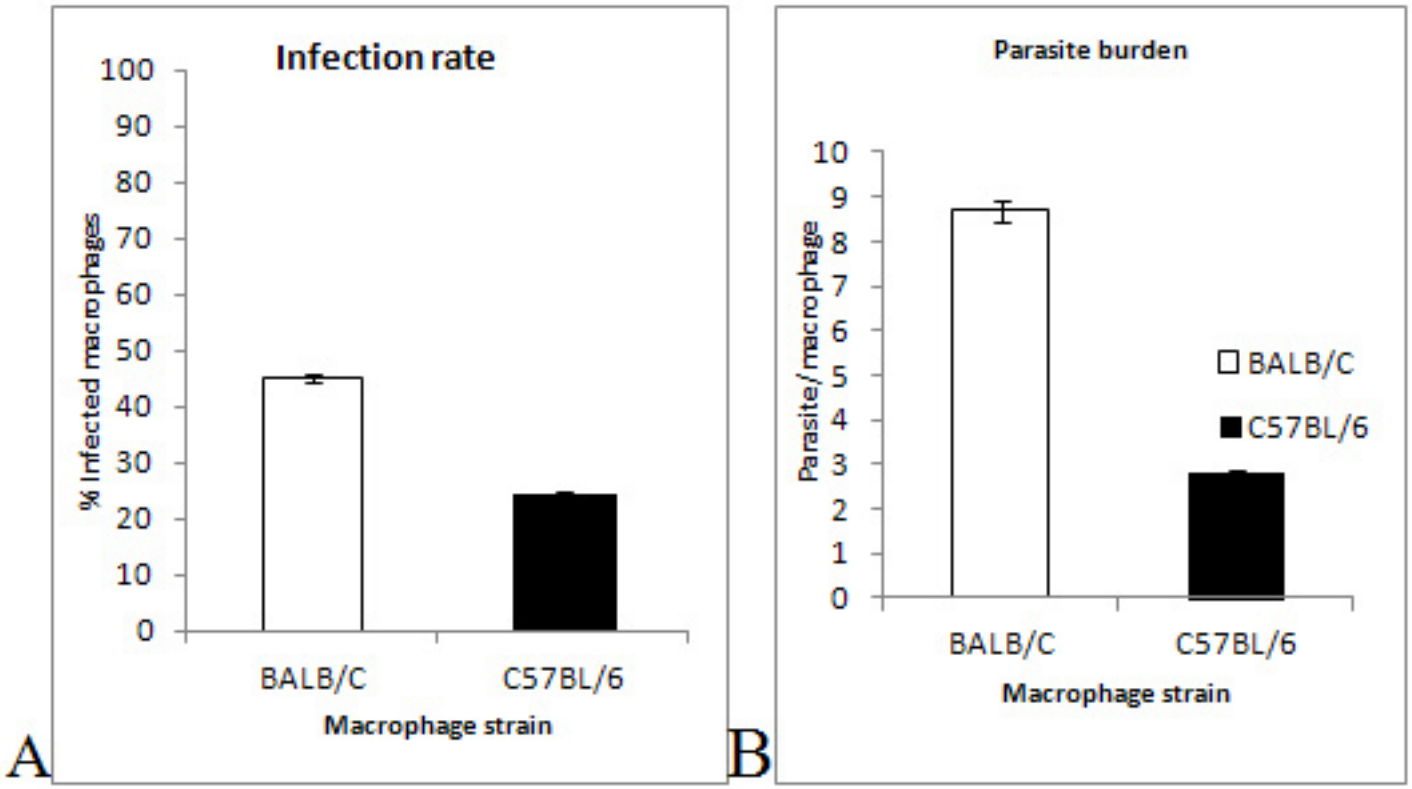


Figure 1: Determination of infection severity: BALB/C and C57BL/6 derived macrophages were challenged with the stationary phase of promastigotes. Smears were taken and stained by Giemsa method from BALB/c (white bar) and C57BL/6 derived macrophages (black bar) for infection severity. (A) Infection rate as the percentage of infected macrophage cells. (B) Parasite burden as the mean number of parasites/ macrophage. Data was shown as mean ± SEM and P£0.05 defined as significant level.

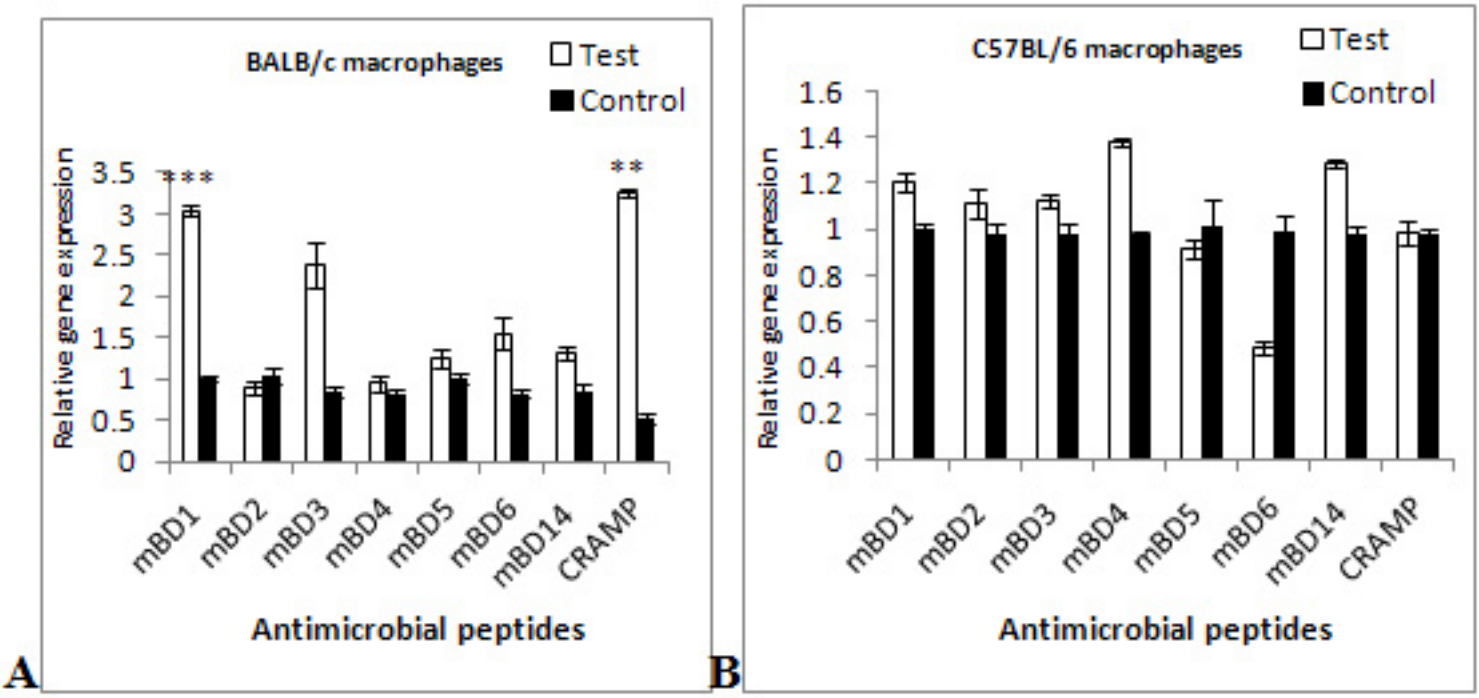
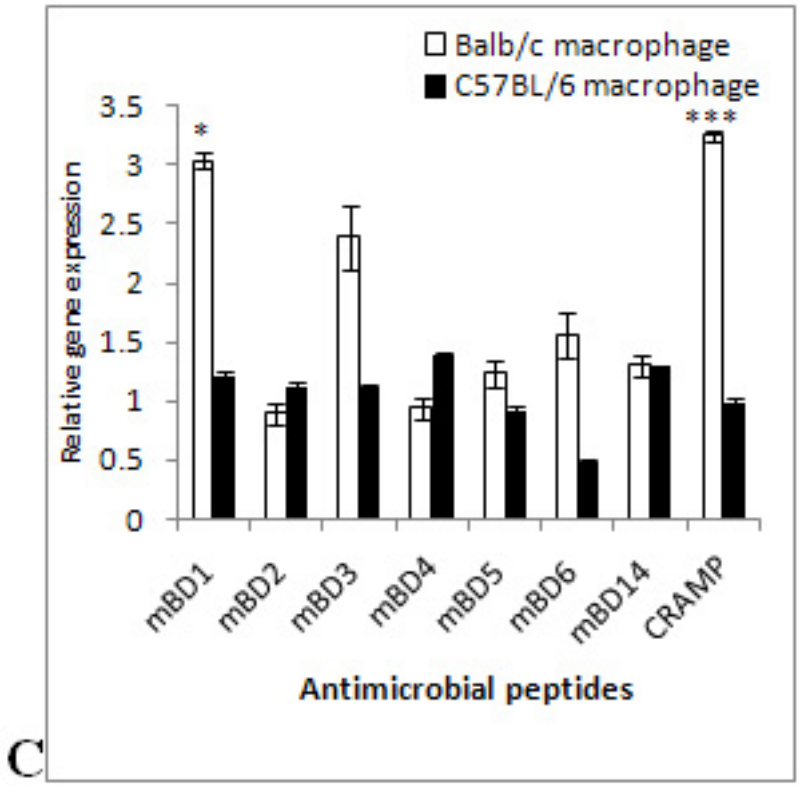


Figure 2. Expression levels of AMPs. BALB/c and C57BL/6 derived macrophages were infected with the stationary phase of *L. major* promastigotes as test groups. Cell sediments were collected 24 hours post infection from test groups (white bar) and controls (black bar) for the expression of selected AMPs. (A) AMPs expression in BALB/c derived macrophages (B) AMPs expression in C57BL/6 derived macrophages. (C) - Next page - Comparison of AMPs expression in both strains. Data are exhibited as means ± SEM and ($p < 0.05$) was defined as significant level.



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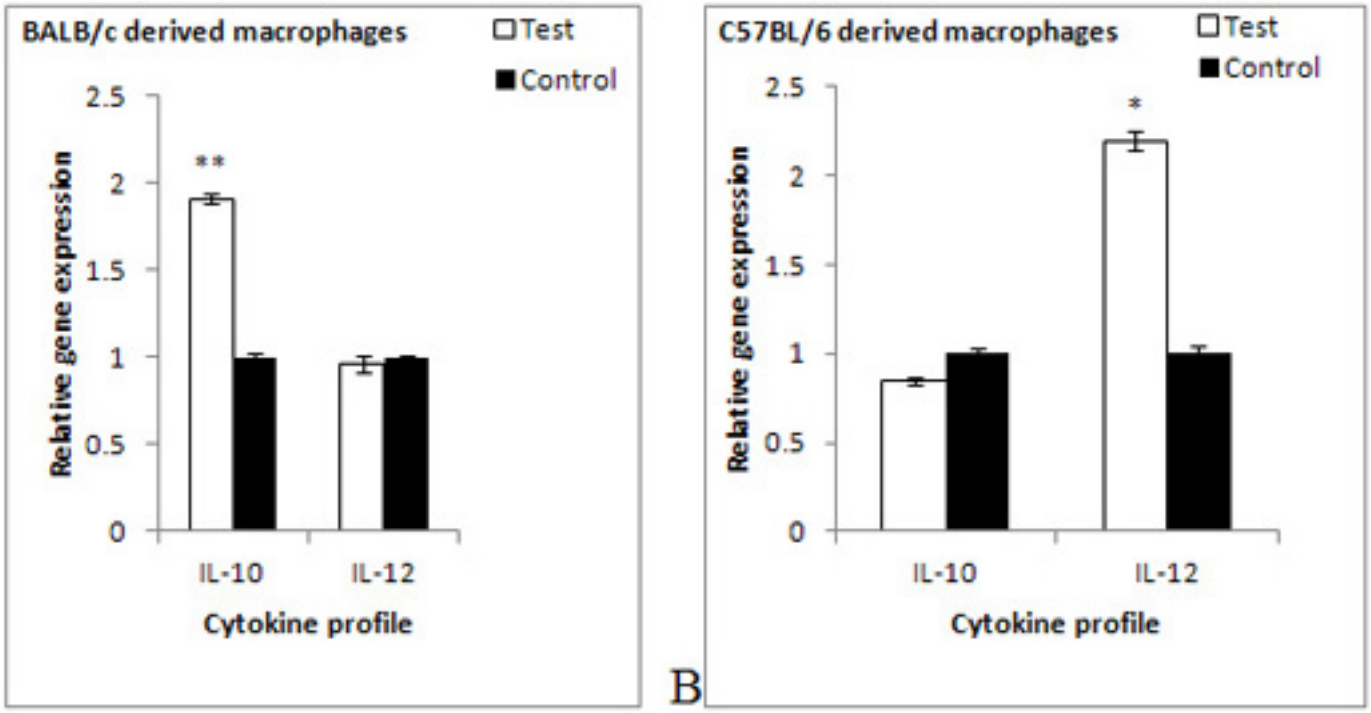

A B

Figure 3. Expression level of cytokines: BALB/c and C57BL/6 derived macrophages were infected with the stationary phase of promastigotes as test groups. Cell sediments were collected 24 hours post infection from test groups (white bar) and controls (black bar) for IL-10 and IL-12 expression. (A) The expression levels of IL-10 and IL-12 in BALB/c derived macrophages. (B) The mRNA levels of IL-10 and IL-12 in C57BL/6 derived macrophages.

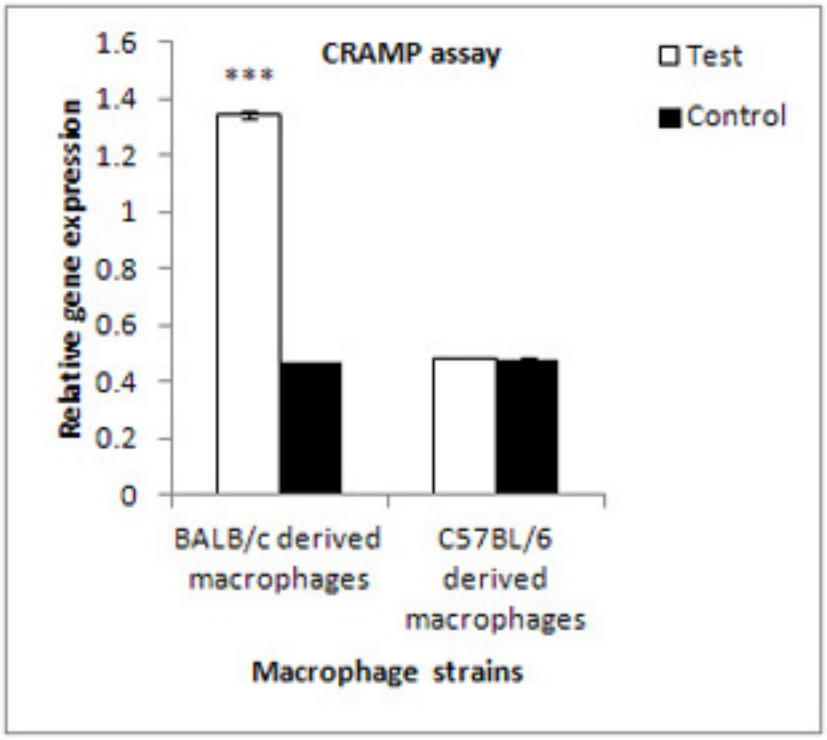

Figure 4

documented studies in this background in the parasitic field. In a described study, human macrophages type I expressed a significant up-regulation of CRAMP between the macrophages types (Bank, 2012). Other research has been generally focused on in vivo models. Radzishevsky et al. (Radzishevsky et al., 2005) showed that CRAMP knock-out gene mice represent a severe infectivity rate of *L. amozonensis* infection in their tissues than wild type. Another aim of this survey was the study of cytokine profiles. Based on the findings related to cytokine profiles, BALB/c derived macrophages expressed a significant up-regulation of IL-10 and a low level of IL-12, while the other type showed completely reverse reaction (Figure 3. A-B). Data from a previous study demonstrated that human derived macrophages type 1 with less sensitivity to *L. major* infection expressed IL-12 more against other types (Bank, 2012). Finally, the information contained in mRNA molecule must be converted to the synthesis of a new protein. Due to higher expression, the newly synthesized peptide of CRAMP was more measured for BALB/c derived macrophages than the other type (Figure 4). Taken together, AMPs consists of a defense barrier against *L. major* infection especially in susceptible macrophages, but cannot create an absolute protection following *L. major* infection.

Acknowledgment

We are grateful from Kerman medical university owing to finance supportive burden of this project and appreciatively the leishmaniasis research center due to use of experience and their requirements.

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