Hirosaki Med. J. 61 (Suppl.) : S238—S244, 2010

THE ROLE OF INTERLEUKIN-17 IN THE PROTECTIVE EFFECT OF AN IMMUNIZATION WITH CLUMPING FACTOR A AGAINST *STAPHYLOCOCCUS AUREUS* INFECTION

Kouji Narita[1, 2], Dong-Liang Hu[1] and Akio Nakane[1, 2]*

Abstract Clumping factor A (ClfA) is an adhesin of *Staphylococcus aureus*, and the residues 40-559 of ClfA ($ClfA_{40-559}$) compose fibrinogen binding domain. It was reported that antibody to $ClfA_{40-559}$ plays an important role in the protective effects. In this study, we investigated the role of IL-17 in the protective effects of an immunization with $ClfA_{40-559}$ against *S. aureus* infection.

Mice immunized or non-immunized with $ClfA_{40-559}$ were challenged with *S. aureus*. Bacterial numbers in the organs and survival rates were evaluated. RAW264.7, DC2.4 and spleen cells of naïve and immunized mice were stimulated with $ClfA_{40-559}$. Cytokine production and mRNA expression in the spleens and cells was determined by ELISA and/or RT-PCR.

The survival rate was improved and the bacterial numbers in the organs were reduced in the mice immunized with $ClfA_{40-559}$. $ClfA_{40-559}$ induced IL-23p19 mRNA expression and IL-6 but not IL-12 production in DC2.4 and RAW264.7 cells. RORγt and IL-17A mRNA expression in the spleen cells of naïve mice was induced. In the spleen cells of immunized mice, $ClfA_{40-559}$ induced the high production of IL-17 but not IFN-γ or IL-4. IL-17A, IL-6 and CXCL2 mRNA expression was also increased in the organs of immunized and challenged mice. These results suggest that an immunization with $ClfA_{40-559}$ induces Th17, and that IL-17 contributes to host defense in early phase of *S. aureus* infection by promoting neutrophil recruitment.

Hirosaki Med. J. 61, Supplement : S238—S244, 2010

Key words: *Staphylococcus aureus*; Clumping factor A; Immunization; IL-17; Th17.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been known a major nosocomial pathogen. Recently, infections caused by community-acquired MRSA have been also reported to increase in healthy children and young adults[1]. Most of clinically isolated strains are resistant to many antimicrobials, leaving few options for effective antimicrobial therapy. There is a need for effective treatment and prevention strategies against MRSA infections, such as immunotherapy.

Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) located on the surface of *S. aureus* have been reported the virulence factor to mediate attachment of bacterial cells to the host. Clumping factor A (ClfA) is a fibrinogen binding MSCRAMM[2] and expressed by virtually all *S. aureus* strains[3]. The structure and biological roles of ClfA have been clarified. The residue region A composed from 40 to 559 contains the fibrinogen binding domain[4,5]. ClfA promotes clumping of *S. aureus* in plasma and adherence of bacterial cells to blood clots, plasma-conditioned biomaterials and human platelets[4,6]. From these studies, ClfA has been indicated an important virulence factor and a potential vaccine candidate.

T helper (Th) cells play an important role in immune responses by exertion of a variety

[1]Department of Microbiology and Immunology, [2]Institute for Animal Experimentation, Hirosaki University Graduate School of Medicine, Hirosaki, Aomori 036-8652, Japan

*Corresponding author. Mailing address: Department of Microbiology and Immunology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori, 036-8562, Japan.

Phone: 81-172-39-5032. Fax: 81-172-39-5034. E-mail: a27k03n0@cc.hirosaki-u.ac.jp.

of effector functions. Th cells have now been classified different three T cell subsets, Th1, Th2 and Th17[7,8]. Interferon-γ (IFN-γ) produced by Th1 cells is involved in immune system to eliminate intracellular pathogens. Cytokines produced by Th2 cells are essential for the humoral immunity to eliminate extracellular pathogens. Th17 cells are a new lineage of $CD4^+$ T cells, which produce interleukin-17 (IL-17)[8,9]. It is suggested that IL-17 plays an important role in host defense against extracellular pathogens by inducing chemokine production and enhancing recruitment of neutrophils[10]. Thus, the functions of cytokine(s) produced by Th cells would be involved in the protective efficacy against *S. aureus* infection.

It was reported that active immunization with A domain of ClfA ($ClfA_{40\text{-}559}$) reduced the severity of staphylococcus-mediated arthritis in mice, and suggested the protective effect is partly mediated by the specific antibody[3]. The important role of antibody in the protection has been also demonstrated in the studies on passive immunization with anti-ClfA antibody[3,11-14]. However, the role of cytokines in the protective effect by an immunization with $ClfA_{40\text{-}559}$ has not been elucidated. In this study, we investigated the role of the cytokines in the protective effect of an immunization with $ClfA_{40\text{-}559}$.

We constructed the plasmid encoding $ClfA_{40\text{-}559}$ and purified the recombinant protein. The $ClfA_{40\text{-}559}$ gene was amplified by PCR using the following PCR primers: forward, 5'-CC CCGAATTCATGAATATGAAGAAAAAA AGAAAAAC-3' and reverse, 5'- CCCCGTC GACTTATTTCTTATCTTTATTTTC-3'. The PCR product was digested with *EcoR*I and *Sal*I, cloned into pGEX-6p-1, and the plasmids were used to transform *Escherichia coli* DH5α cells. Expression and purification of recombinant $ClfA_{40\text{-}559}$ were preformed as described elsewhere[15].

Activation of dendritic cells and macrophages results in the production of a variety of cytokines that are involved in the differentiation of Th cells. To investigate the ability of $ClfA_{40\text{-}559}$ to induce cytokine production in dendritic cells and macrophages, murine macrophage cell line RAW264.7 and murine dendritic cell line DC 2.4 were used in this sturdy. RAW264.7 and DC2.4 were purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). RAW264.7 was cultured in DMEM medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% of fetal bovine serum (JRH Biosciences, Lenexa, KS, USA) and 3% L-glutamine (Wako Pure Chemical Industries, Osaka, Japan). DC2.4 was cultured in RPMI 1640 medium (Nissui) supplemented with 10% of fetal bovine serum and 1% L-glutamine. Cells at 2×10^6/ml were incubated at 37°C in the presence or absence of 10 μg/ml $ClfA_{40\text{-}559}$. The cell culture supernatant was collected 48 h after incubation. The amounts of IL-12p70 in the supernatants of cell cultures were determined by double-sandwich enzyme-linked immunosorbent assays (ELISAs) as described previously[16-18], and the amounts of IL-6 in the supernatants were determined by ELISA, using mouse IL-6 Cytoset™ kit (Biosource USA, Camarillo, CA, USA). Total mRNA was extracted from spleen cells 4 h after incubation, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNAs were synthesized by reverse transcription of 1 μg total RNA using random primers (Takara, Shiga, Japan) and reverse transcriptase Moloney murine leukemia virus (Invitrogen). To assess IL-23p19 mRNA expression, the following primers were used, forward, 5'-CCTGGCTGTGCCTAGGAGTAGC-3', and reverse, 5'-AAAAGCCAGACCTTGGCGGATC C-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward, 5'-TGAAGGTCGGTGTG AACGGATTTGG-3', and reverse, 5'-ACG ACATACTCAGCACCAGCATCAC-3'. Gene expression levels were determined by real-time PCR analysis using SYBR Green Supermix

(BIO-RAD Laboratories, Hercules, CA, USA). Dissociation curves were used to detect primer-dimer conformation and nonspecific amplification. The threshold cycle (C_T) of each target product was determined and set in relation to the amplification plot of GAPDH. The detection threshold is set to the log linear range of the amplification curve and kept constant (0.05) for all data analysis. Difference in C_T values (ΔC_T) of two genes was used to calculate the relative expression: relative expression = $2^{-(C_T \text{ of target gene} - C_T \text{ of GADPH})} = 2^{-}\Delta^{C}_{T}$. In RAW 264.7, IL-6 production and IL-23p17 mRNA expression of cells stimulated with $ClfA_{40\text{-}559}$ was 200 times and 5 times increased compared with those of non-stimulated cells, respectively. In DC2.4, IL-6 production and IL-23p17 mRNA expression of cells stimulated with $ClfA_{40\text{-}559}$ was 100 times and 2.2 times increased compared with those of non-stimulated cells, respectively. IL-12p70 was not detected in both the stimulated and non-stimulated cells by ELISA. Thus, IL-6 but not IL-12p70 production and IL-23 p19 mRNA expression in the RAW 264.7 and DC 2.4 were significantly enhanced by stimulation with $ClfA_{40\text{-}559}$. In this study, statistical analyses of cytokine titers and relative expression of mRNA were made via Student's *t*-test. Cytokine profiles, which are involved in the differentiation of Th cells, were also evaluated in spleen cells of naïve BALB/c mice in vitro. BALB/c mice were purchased from Clea Japan, Tokyo, Japan and maintained under specific pathogen-free conditions at the Institute for Animal Experimentation, Hirosaki University Graduate School of Medicine. Food and water were given ad libitum. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Hirosaki University. The removal of spleens from the mice was performed on day 7 after the last booster, and spleen cells were obtained as described previously[15]. The spleen cells were placed in 24-well tissue culture plate at density of 2×10^6 cells/well and incubated in RPMI 1640 medium supplemented with 10% of fetal bovine serum, 1% L-glutamine, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. Ten μg/ml $ClfA_{40\text{-}559}$ was added to stimulate the cells. The supernatants were collected 48 h after incubation, and the cytokines produced in the in the supernatants of cell cultures were assessed. The amounts of IL-4 were determined by double-sandwich enzyme-linked immunosorbent assays (ELISAs) as described previously[16-18]. IL-6 production of cells stimulated with $ClfA_{40\text{-}559}$ was 120 times increased compared with that of non-stimulated cells. IL-12p70 and IL-4 production of both stimulated and non-stimulated cells were not detected by ELISA. To assess mRNA expression, cells were collected 4 h after incubation and real time quantitative PCR analyses were performed. IL-23p19, TGF-β[19], IL-21[20] and RORγt[21] mRNA expression levels were also significantly increased 4 h after stimulation. IL-6, IL-21 and TGF-β are cytokines that are involved in differentiation of Th17 cells[22]. IL-23 maintains and extends Th17 phenotype and to promote survival of these cells[22,23]. It was also reported that RORγt is the key transcription factor that orchestrates the differentiation of Th17 cells[24]. These results demonstrated that molecules involved in differentiation Th0 to Th17 are induced by stimulation with $ClfA_{40\text{-}559}$.

Next, cytokine profiles produced by spleen cells of immunized and non-immunized control mice were assessed. For immunization, $ClfA_{40\text{-}559}$ was dissolved in PBS and emulsified 1:1 in alum adjuvant (Pierce, Rockford, IL, USA), and mice were subcutaneously injected with 200 μl of the emulsion containing 10 μg of the recombinant protein or PBS plus adjuvant, and booster immunization was performed on 14 and 28 days after the initial immunization. The amounts of IFN-γ in the supernatants of cell cultures were determined by ELISA as described previously[16-18].

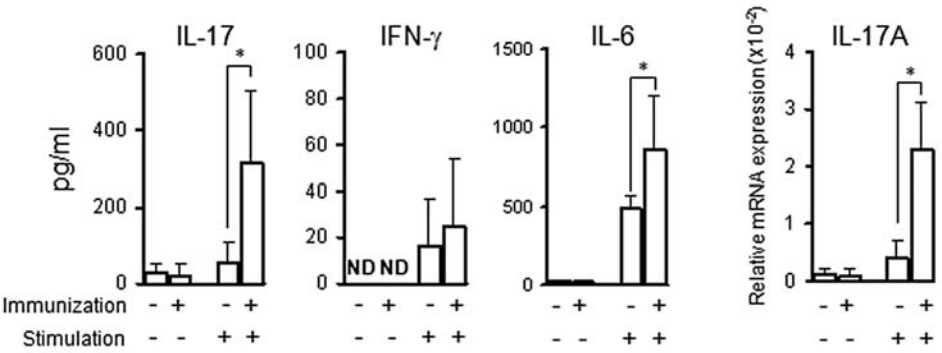

Figure 1 Cytokine production and mRNA expression in the spleen cells of immunized and control mice. Mice were immunized or non-immunized as described in the text. Spleen cells were incubated in the absence or presence of 10 μg/ml $ClfA_{40\text{-}559}$ for 48 h. Levels of IL-17, IFN-γ and IL-6 in the supernatants of cell cultures were determined by ELISAs. Spleen cells of immunized and control mice were incubated in the absence or presence of 10 μg/ml $ClfA_{40\text{-}559}$ for 4 h. The level of relative mRNA expression of IL-17A was determined by real-time quantitative RT-PCR. Data are expressed as the means ± SD for a group of six to eight mice. ND indicates not detectable. An asterisk represents a statistically significant difference from the control at $P < 0.05$

The amounts of IL-17 in the supernatants were determined by ELISA, using Mouse IL-17 ELISA (Bender MedSystems GmbH, Venna, Austria). IL-6, IL-17 production and IL-17A mRNA[25] expression were significantly increased in the spleen cells of immunized mice by stimulation with $ClfA_{40\text{-}559}$ (Figure 1). In contrast, an immunization with $ClfA_{40\text{-}559}$ showed no effect on IFN-γ production, and IL-4 was not detected by ELISA in the culture supernatants. Because IFN-γ and IL-4 are main cytokines produced by Th1 and Th2 cells, respectively, these results suggest that Th17 cells are induced by an immunization with $ClfA_{40\text{-}559}$.

To evaluate the protective effect of an immunization with $ClfA_{40\text{-}559}$, *S. aureus* 834 strain, a clinical septic isolate, was used for infection of mice in this study. The bacteria were grown in tryptic soy broth (BD Diagnosis Systems, Sparks, MD, USA), harvested by centrifugation and washed with sterile PBS. Mice were challenged at 5×10^7 CFU/mouse by intravenous injection 7 days after the last booster, and survival rates of mice and bacterial numbers in the organs were determined[15]. The survival rate of immunized mice was significant higher than the control mice (Figure 2A). The bacterial numbers in the spleens and livers were reduced on day 3, and those in the kidneys of immunized mice were significantly reduced on days 1 and 3 (Figure 2B). Statistical analyses of bacterial counts were made via Student's *t*-test. For survival experiments, the Kaplan–Meier method was used to obtain survival fractions, and significance was determined by a log rank test. $P<0.05$ was considered as a significance. The important role of antibody in the protection has been demonstrated in the previous studies on passive immunization with anti-ClfA antibody[3,11-14]. Hence, we assessed specific antibody levels in the sera of immunized and control mice. The serum samples were obtained from immunized and control mice 7 days after last immunization and diluted twofold with 10% Blockace (Dainippon Pharmaceutical Co. Ltd. Osaka, Japan) in PBS. The titers of antibodies were determined by ELISAs as described previously[15] and defined as the highest dilution giving absorbance value of more than twice that of 10% Blockace in PBS. The levels of anti-$ClfA_{40\text{-}559}$ IgG1, IgG2a, IgG2b and IgG3 antibodies in the sera of immunized mice were 2^{25}, 2^{17}, 2^{15}, 2^{10}, respectively, while the titers of non-immunized mice were less than 2^5.

We next investigated IL-17-mediated

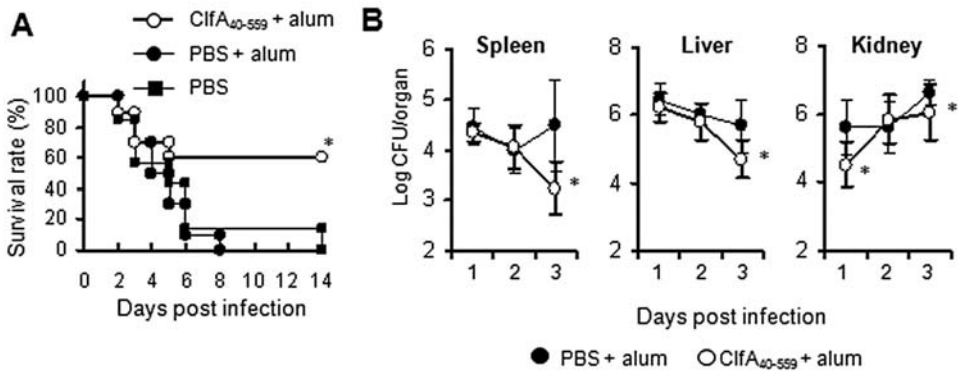

Figure 2 Protection by an immunization with ClfA40-559 against *S. aureus* infection. Immunized and control mice were infected with *S. aureus* intravenously. (A) Survival rates of immunized and control mice. Mice were immunized with ClfA40-559 plus alum or administered PBS plus alum or PBS only as the control. The animals were monitored for 14 days after challenge. An asterisk represents a statistically significant difference from the PBS plus alum administered control at $P < 0.05$. (B) Bacterial numbers in the spleens, livers and kidneys of immunized and control mice. The organs of mice were obtained 1, 2 and 3 days after challenge. Each result represents the mean ± SD for a group of eight to ten mice.

protection against *S. aureus* infection. The immunized and non-immunized mice were challenged with *S. aureus* and the spleens were obtained 24 and 48 h after challenge. The IL-17A mRNA level was increased in the spleens of immunized mice 24 h but not 48 h after challenge, the relative mRNA expression 24 h after challenge in the immunized and non-immunized mice were 2×10^{-4} and 0.3×10^{-4}, respectively. In addition to IL-17A, CXCL2[26] and IL-6 mRNA expression was significantly increased 48 h after infection in the immunized mice, CXCL2 mRNA expression of immunized and non-immunized mice were 1.5×10^{-2} and 0.5×10^{-2}, and IL-6 mRNA expression were 7×10^{-2} and 2.2×10^{-2}, respectively. CXCL2 and IL-6 are known to promote neutrophil recruitment[27]. IL-17 has been shown to regulate the production of CXCL1, CXCL2 that induce emigration of neutrophils into infection sites[27-29], and also regulate IL-6 that is involved in neutrophil recruitment. Previous studies demonstrated that IL-17 is critical for optimal production of CXCL2 and plays an important role in host defense against *Klebsiella pneumoniae* infection[30], and that IL-17 enhances neutrophil recruitment and contributes lung defense against *Mycoplasma pneumoniae* infection[31]. IL-17A and IL-17F are important for host defiance against *S. aureus* infection[32], and neutrophils are pivotal for elimination of the pathogen[33,34]. The present study showed that IL-17A mRNA expression was increased 24 h after bacterial challenge in the spleens of immunized mice, and CXCL2 and IL-6 mRNA expression was increased after IL-17A expression. These results suggest that IL-17 might be involved in neutrophil recruitment in spleen.

The present study demonstrated that an immunization with $ClfA_{40-559}$ induces, in addition to the specific antibody, IL-17 production by Th17 cells in the early phase of *S. aureus* challenge. IL-17 may contribute to elimination of *S. aureus* by enhancement of neutrophil recruitment in the infection foci. These findings are useful for development effective vaccines against MRSA infection.

References

1) Weber JT. Community-associated methicillin-resistant *Staphylococcus aureus*. Clin Infect Dis 2005; 41 Suppl 4:S269-72.

2) Foster TJ, Hook M. Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol 1998;6:484-

8.

3) Josefsson E, Hartford O, O'Brien L, Patti JM, Foster T. Protection against experimental *Staphylococcus aureus* arthritis by vaccination with clumping factor A, a novel virulence determinant. J Infect Dis 2001;184:1572-80.

4) McDevitt D, Francois P, Vaudaux P, Foster TJ. Identification of the ligand-binding domain of the surface-located fibrinogen receptor (clumping factor) of *Staphylococcus aureus*. Mol Microbiol 1995;16:895-907.

5) Hartford O, Francois P, Vaudaux P, Foster TJ. The dipeptide repeat region of the fibrinogen-binding protein (clumping factor) is required for functional expression of the fibrinogen-binding domain on the *Staphylococcus aureus* cell surface. Mol Microbiol 1997;25:1065-76.

6) Siboo IR, Cheung AL, Bayer AS, Sullam PM. Clumping factor A mediates binding of *Staphylococcus aureus* to human platelets. Infect Immun 2001;69:3120-7.

7) Reiner SL. Development in motion: helper T cells at work. Cell 2007;129:33-6.

8) Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005;6:1123-32.

9) Park H, Li Z, Yang XO, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005; 6:1133-41.

10) Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 2008;28:454-67.

11) Hall AE, Domanski PJ, Patel PR, et al. Characterization of a protective monoclonal antibody recognizing *Staphylococcus aureus* MSCRAMM protein clumping factor A. Infect Immun 2003;71:6864-70.

12) Domanski PJ, Patel PR, Bayer AS, et al. Characterization of a humanized monoclonal antibody recognizing clumping factor A expressed by *Staphylococcus aureus*. Infect Immun 2005;73:5229-32.

13) Vernachio J, Bayer AS, Le T, et al. Anti-clumping factor A immunoglobulin reduces the duration of methicillin-resistant *Staphylococcus aureus* bacteremia in an experimental model of infective endocarditis. Antimicrob Agents Chemother 2003;47:3400-6.

14) Patti JM. A humanized monoclonal antibody targeting *Staphylococcus aureus*. Vaccine 2004;22 Suppl 1:S39-43.

15) Hu DL, Omoe K, Sasaki S, et al. Vaccination with nontoxic mutant toxic shock syndrome toxin 1 protects against *Staphylococcus aureus* infection. J Infect Dis 2003;188:743-52.

16) Nakane A, Okamoto M, Asano M, Kohanawa M, Minagawa T. Endogenous gamma interferon, tumor necrosis factor, and interleukin-6 in *Staphylococcus aureus* infection in mice. Infect Immun 1995;63:1165-7.

17) Nakane A, Nishikawa S, Sasaki S, et al. Endogenous interleukin-4, but not interleukin-10, is involved in suppression of host resistance against *Listeria monocytogenes* infection in gamma interferon-depleted mice. Infect Immun 1996;64:1252-8.

18) Miura T, Kudo T, Matsuki A, et al. Effect of 6-hydroxydopamine on host resistance against *Listeria monocytogenes* infection. Infect Immun 2001;69:7234-41.

19) Nakane A, Asano M, Sasaki S, et al. Transforming growth factor beta is protective in host resistance against *Listeria monocytogenes* infection in mice. Infect Immun 1996;64:3901-4.

20) Brandt K, Bulfone-Paus S, Foster DC and Ruckert R. Interleukin-21 inhibits dendritic cell activation and maturation. Blood 2003;102:4090-8.

21) Kim MY, Toellner KM, White A, et al. Neonatal and adult CD4+ CD3- cells share similar gene expression profile, and neonatal cells up-regulate OX40 ligand in response to TL1A (TNFSF15). J Immunol 2006;177:3074-81.

22) Tato CM, O'Shea JJ. Immunology: what does it mean to be just 17? Nature 2006;441:166-8.

23) Baran J, Guzik K, Hryniewicz W, Ernst M, Flad HD, Pryjma J. Apoptosis of monocytes and prolonged survival of granulocytes as a result of phagocytosis of bacteria. Infect Immun 1996;

64:4242-48.

24) Ivanov, II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17^{+} T helper cells. Cell 2006;126:1121-33.

25) Raffatellu M, Santos RL, Chessa D, et al. The capsule encoding the viaB locus reduces interleukin-17 expression and mucosal innate responses in the bovine intestinal mucosa during infection with *Salmonella enterica* serotype Typhi. Infect Immun 2007;75:4342-50.

26) Ikejima S, Sasaki S, Sashinami H, et al. Impairment of host resistance to *Listeria monocytogenes* infection in liver of db/db and ob/ob mice. Diabetes 2005;54:182-9.

27) Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity 2004;21:467-76.

28) Aujla SJ, Dubin PJ, Kolls JK. Th17 cells and mucosal host defense. Semin Immunol 2007;19:377-82.

29) Witowski J, Pawlaczyk K, Breborowicz A, et al. IL-17 stimulates intraperitoneal neutrophil infiltration through the release of GRO alpha chemokine from mesothelial cells. J Immunol 2000;165:5814-21.

30) Ye P, Rodriguez FH, Kanaly S, et al. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J Exp Med 2001;194:519-27.

31) Wu Q, Martin RJ, Rino JG, Breed R, Torres RM, Chu HW. IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory *Mycoplasma pneumoniae* infection. Microbes Infect 2007;9:78-86.

32) Ishigame H, Kakuta S, Nagai T, et al. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity 2009;30:108-19.

33) Molne L, Verdrengh M, Tarkowski A. Role of neutrophil leukocytes in cutaneous infection caused by *Staphylococcus aureus*. Infect Immun 2000;68:6162-7.

34) Verdrengh M, Tarkowski A. Role of neutrophils in experimental septicemia and septic arthritis induced by *Staphylococcus aureus*. Infect Immun 1997;65:2517-21.