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**SPECIAL ARTICLE**

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# STAPHYLOCOCCAL SUPERANTIGEN C IN RHEUMATOID ARTHRITIS PATIENT'S BLOOD AND SYNOVIAL FLUID: A COMPARATIVE STUDY

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## ABSTRACT

**Introduction and aim:** Staphylococcal enterotoxins C as superantigen could stimulate T lymphocytes and trigger a cytokine storm and subsequently Rheumatoid arthritis (RA) as an autoimmune disease. The aim of this study was to assess the existence of staphylococcal enterotoxin C in Rheumatoid Arthritis patients' blood and synovial fluid (SF) samples.

**Materials and Methods**: During 18 months, patients whom were referred to Rheumatologic clinic and with an initial diagnosis of Rheumatoid arthritis, 100 blood and 100 synovial fluid samples were collected in a volume of 5-10ml, 3-5ml of which was inoculated to Castaneda medium, and the remained was collected in the test tubes for obtaining serum and plasma. PCR and ELISA methods by using specific primer and special Antibodies for detecting enterotoxin C were carried out.

**Results:** During this study and after sequential sub cultures, only 2 bacterial were isolated. Based on the results of Biochemical tests, just one case as Staphylococcus aureus was detected. Also, the result of molecular diagnosis of enterotoxin C gene was 39.75% and about the ELISA test the result was 37.34%.

**Conclusion:** The role of Bacterial superantigens was considered in the pathogenesis of Rheumatoid arthritis, but it's origin is unknown. Regardless of



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whether it is endogenous or exogenous origin may be provide a good model for the diagnosis and treatment of disease. However, further study is needed the finding of this study might be most important in related pathophysiology of RA disease.

## INTRODUCTION

The involvement roles of staphylococcal superantigens (enterotoxins) in inflammatory diseases such as atopic dermatitis, Kawasaki syndrome, nasal polyposis and Rheumatoid Arthritis (RA) have been reported (1-3). The popularity of the staphylococcal enterotoxins was pertaining to toxic shock syndrome and food poisoning (4). While, understanding the superantigenic characteristics activity, and their roles in inflammatory disease, has encouraged the researchers for more investigation. The results of several researches revealed that *Staphylococcus aureus* enterotoxins can cause diseases by inducing high levels of pro-inflammatory cytokines from CD4+ T cells and antigen-presenting cells, which are activated through binding of wild-type superantigens to both the MHC class II molecule and specific T-cell receptor Vbeta chains (5). Although, several research results indicated that, the family of staphylococcal superantigens has been known to contribute to a broad-spectrum of diseases (6,7). While, our previous studies results indicated that, staphylococcal enterotoxins in SF samples of patients with RA were tracked (8). In addition, The most of the research by using PCR method was rely on the isolated of *S. aureus* strains and then they have been shown one or more of the staphylococcal superantigen gene incoding in SF of patients with RA (9,10). Furthermore, Staphylococcal *ent*C gene in synovial fluid of patients with rheumatoid arthritis was reported for the first time (11). But the presence of these genes in the same patients' blood is not clarified. However, unanswered questions were remained. Dose the equivalency of presence of *ent*C gene in SF and blood samples of patients with RA? In the samples that *ent* C gene has been detected, whether the enterotoxin C proteins is detectable. To fine the answers of the questions, this research was conducted. The aim of this study was to compare of three methods for diagnosis of Staphylococcal superantigen C in Rheumatoid arthritis Patient's Blood and Synovial fluid

## Materials and methods

**Bacterial standard strain:** A confirmative producing *S.aureus* strain which derived from the clinical isolate and characterized for enterotoxin C gene was used in this study as standard strain (12).

**Primer Design:** All primer pairs were used based on previously published (13) which designed using standard sequence analysis software. In addition, multiple alignments were carried out by DNASIS MAX trial version. This primer pair was amplified a 102bp fragment.

### Blood and Synovial fluid Sample collection

The study protocol approved by the Ethical Committee of the Baqiyatallah University of Medical Sciences, Teheran, IR Iran, on November 29, 2012, with Code No: 24, Paragraph 28.

A total of 100 Rheumatoid Arthritis patients' blood and synovial fluid samples from April 2013 to August 2015 were referred to laboratory by Rheumatologists. Based on ACR 2010 criterions, inclusion and exclusion criteria of the Rheumatoid Arthritis patients were selected (14).

The act of sampling based on Microbial Standard Protocol and by perfect observance of Aseptic conditions has been done. Then, amount of 5-10 ml blood sample was aspirated. As the next step, 3-5 ml of blood was immediately inoculated into Castaneda medium and has been incubated at 37°C for 48 hours. And finally, the remained of blood has been added to sterile CBC tube and the clotting one.

### Blood culture

After inoculating to Castaneda medium and incubation, the amount of 1 ml of Castaneda medium by syringe were aspirated and sub culture on Blood agar medium was done. After incubation at 37°C and for 24 hours, base on colony characteristics, Gram stain, catalase, oxidase, motility, sugar fermentation, susceptibility to Bacitracin and Novobiosin, coagulase and DNase tests the isolated bacterial have been examined.

### DNA Extraction from bacteria

For the PCR method, first of all, the bacterial standard strain was inoculated into the 5 ml LB Broth medium and incubated at 37°C for 24 hours. And then bacterial suspensions were centrifuged, and cellular sediments were obtained and base on Salting out Modified method the DNA has been extracted (15).

### DNA Extraction from Blood Buffy Coat

In aseptic condition and carefully, patient's blood has been added to CBC tube and was centrifuged (10 min, 7000×g in 4°C). Then blood's Buffy coat were selected and transferred to DNA free sterile tube. Separately 100 µl of each of the blood′s Buffy coat genomes has been extracted by CinnaPure DNA Extraction Kit (Cat. NO. PR881612, Cinnagen Co. Iran). Based on the Kit instructions as briefly, the amount of 100 µl of blood's Buffy coat has been added to the Kit's micro tube, then 400 µl of Lysis Buffer has been added to it and for 20 seconds has been vortexed, then 300 µl of Precipitation Solution has been added to micro tube and again has been vortexed for 5 seconds. In this stage whole of the micro tube components has been transferred to the column, and has been centrifuged (1 min, 5°C, 13000×g). Then, the column has been transferred to the new micro tube and has been washed by 400 µl of Washing Buffer 1, and again has been centrifuged (1 min, 5°C, 12000×g), and the second time, the column has been transferred to the other new micro tube and washed by 400 µl of Washing Buffer 2, and again has been centrifuged (1 min, 5°C, 12000×g). After drying and changing the column, the amount of 30 µl of Elution Buffer has been added and has been centrifuged (5 min, 12000×g), finally the DNA quality and quantity has been measured by NanoDrop (Thermo Scientific NanoDrop 2000 Spectophotometer USA). Similarly, the serum and plasma of patients were subjected to DNA extraction by the method.

**DNA Extraction from Synovial Fluid**

Free DNA tubes containing 500 µl of patient's SF and 500 µl of free DNA Distilled water (DW) (mixed in aseptic conditions) were centrifuged for 1 min at 3000×g at 4°C. Afterwards, the resulting supernatants were transferred to DNA free sterile tubes and the genomes were extracted with the CinnaPure-DNA Extraction Kit (Cat. NO. PR881612, Cinnagen Co., Teheran, Iran). Based on the kit instructions, the amount of 200 µl of diluted SF has been added to the kit's micro tube, then 400 µl of Lyses Buffer were added to it and the mixture has been vortexed for 20 seconds. Afterwards, 300 µl of precipitation solution have been added to the micro tubes and was vortexed again for 5 seconds. During this stage, whole of the micro tubes contents were transferred to the column and centrifuged for 1 min at 13000×g at 5°C. The column was afterwards transferred to the new micro tube and washed by 400 µl of Washing Buffer 1, and again centrifuged for 1 min at 12000 g at 5°C. The resulting solution has been transferred to the other new micro tube and washed by 400 µl of Washing Buffer 2, and again centrifuged for 1 min at 12000×g at 5°C. After drying and changing the column, the amount of 30 µl of Elution Buffer was added and the solution centrifuged for 5 min, at 12000×g at 5°C. Finally, in each case, the DNA quality and quantity were measured with the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

**Polymerase Chain Reaction**

For the diagnosis of enterotoxin C gene in Rheumatoid arthritis Patient's Blood, by using the specific primer pairs which amplified a 102bp fragment and the standard strain genome, the PCR method has been set up.

For DNA amplification, the mastermix component was made in 200 µl microtubes by using a 25 µl reaction mixture that contained DNA template as a 3 µl, and 0.5 U of Taq DNA polymerase as the process enzyme, plus 2.5 µl of 10X PCR buffer and also contained 0.4 mM of dNTPs, 3 mM MgCl2 (all reagents were from Cinnagen), 10 pmol of the primer pairs (synthesized by cinnagen) and also 14.5 µl double-distilled water, to a total volume of 25 µl. All of component were carried out in a thermal cycler (Bio-Rad, C1000) with initial denaturation zone at 95°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 50.5°C for 30 seconds and extension at 72°C for 40 seconds, followed by a final extension at 72°C for 5 minutes. Then PCR products have been electrophoresed in a 2% agarose gel for 45 minutes, and with ethidium bromide been stained for 20 minutes (0.05 mg/ml; Sigma Aldrich). So, under ultraviolet light using Gel Doc the gels were photographed (Bio Rad UniversalHood II, USA). Also, in each agarose gel Molecular size markers (50 and 100 bp) have been included. And then all patients' buffy coat genomes by the optimization PCR have been assayed.

**Enzyme-linked Immunosorbent Assay**

For the purpose of validation of the enterotoxin C existence in patients' blood and synovial samples, the indirect ELISA has been supposed. So, CBC tube has been centrifuged (10 minutes with 7000 xg) and patients' plasma was taken out and evaluated by ELISA as follows: Based on the previous research (8), 50 µl of patients' plasma transferred to micro tube and with the 50 µl of Phosphat Salin Buffer as Diluents have been mixed. Then, all of the components were added to the

difind wells of ELISA plates that were coated with polyclonal monospecific antibody against staphylococcal enterotoxin C (Rb PAb enterotoxin C Ab 15899 500 lot 722163; from Abcam Germany). In this stage wells' component were incubated (one hour) and washed ( three times), and so, 50 µl the conjugate antibody was added. Then Enzyme substrate was added, finally the amount of 100 µl stopping solution has been added. After 10 minutes of this process the component's color were changed. After that, the wells absorption by the ELISA reader device in 450 nm wavelength was measured and then for each of the plates the Cut off value has been computed.

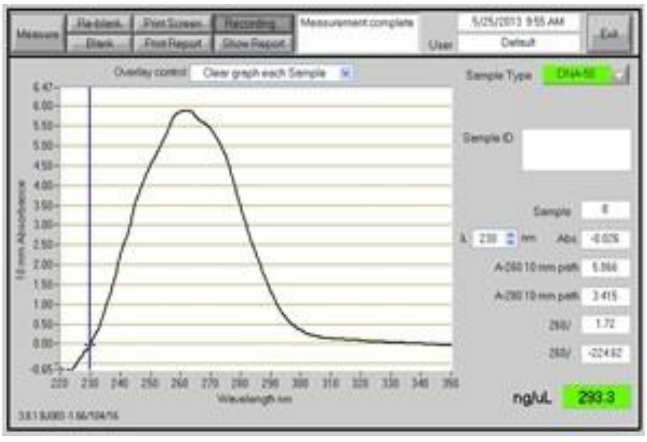
## RESULTS

### The result of Microbial culture on Blood Agar

A total of 100 blood and 100 synovial samples during April 2013 to August 2015 were assessed from Rheumatoid Arthritis patients. During this research and after sequential sub cultures only 2 cases were positive for staphylococcal strains growth, Based on the Biochemical tests just one of them as *Staphylococcus aureus* have been detected and others as *S.intermedius* have been diagnosed.

### The result of DNA extraction

By both Gel electrophoresis (the gel concentration 0/8%, in 100 Volt for 45 min) and Nono drop device the quality and quantity of the standard strain and patients sample extracted genomes have been examined (Fig 1). In compare, both results were relieable.



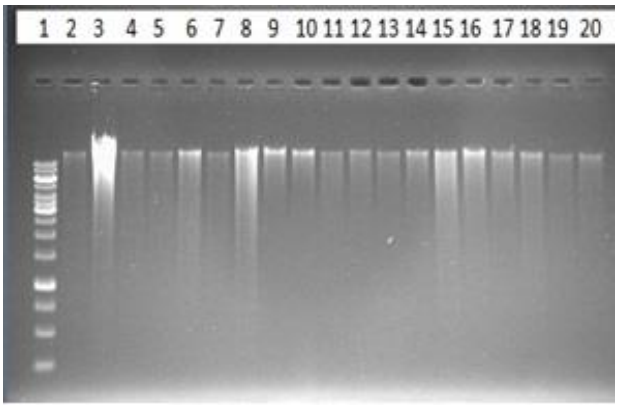




**Figure 1.** on the left), the result of Nono drop DNA evaluation; on the right) electrophoresis result of DNA extraction from blood Buffy coat samples is shown. Line 1 is 100bp Molecular Marker; Lines 2 to 20 are DNA that has been extracted from patient's blood.

### The result of rheumatoid arthritis patients' blood PCR

PCR has been set up after some examinations based on the standard strain genome and master mix components and as a positive control were applied for each test (Fig 2).

The results of primer designing were as; F-TGTATGTATGGAGGTGTAAC and R-AATTGTGTTTCTTTTATTTTCATAA which was amplified a 102 bp amplicons as PCR products.

As it shown in figure 2 the results of samples' PCR with amplifier primer of a 102 bp fragment of staphylococcal ent C with frequency 39.75% was obtained

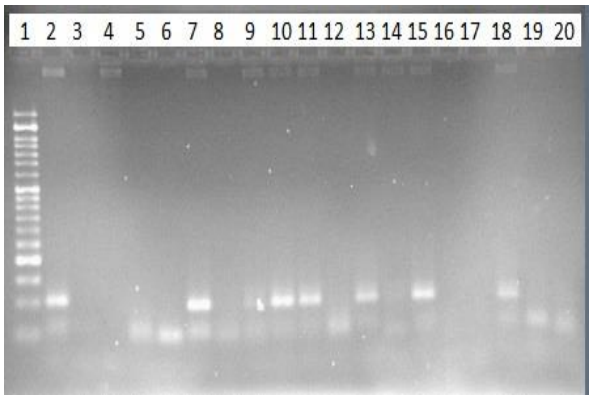



**Figure 2.** an example of the result of PCR amplicon 102 bp. Line 1 is a 50 bp MW standard, line 2 is the standard strain (positive control), lines 7, 9, 10, 11, 13, 15, and 18 have shown the 102 bp and line 20 is negative control.

### The result of ELISA

The results of ELISA plates indicated that 37.34% of rheumatoid arthritis blood (bufy coat) was

positive for staphylococcal enterotoxin C (Fig 3). The comparative results of PCR, ELISA and bacterial culture were illustrated in fig 4.

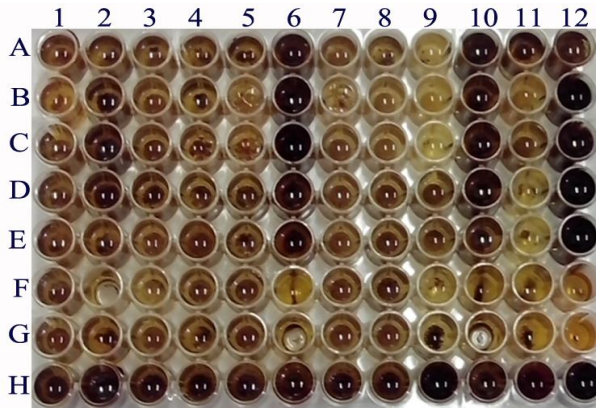



**Figure 3.** The results of ELISA Plate for staphylococcal enterotosin C were shown. The vertical rows A-E are patient's number samples and the horizontal rows F and G are negative controls and H raw is the positive control. All wells with burned brown color have at least equal optical density or more than H rows (positive controls) are considered as positive for staphylococcal enterotosin C.

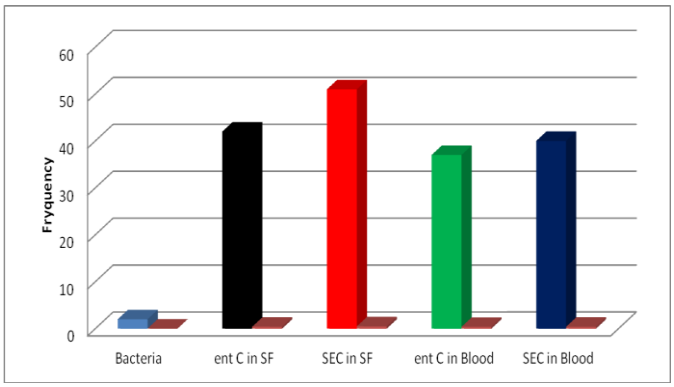



**Figure 4.** comparative results of PCR, ELISA and bacterial isolation is shown. Staphylococcal enterotoxin C assay of 100 rheumatoid arthritis patients' blood and synovial samples.

## DISCUSSION

Arthritogenic potential of Staphylococcal superantigen A (SEA) and B (SEB) was reported (16, 17). In addition, in order to show the possible role of staphylococcal superantiges in Rheumatoid arthritis, a series of research was design and conducted. The results of those investigations indicate the existence of staphylococcal superantiges in synovial fluids (18,19). However, the origin of the superantigens existence in synovial fluids is unknown. Furthermore, the difficulty in preparation of the synovial fluids aspiration from the patients is the main challenges and causes the patients more harassment. For this reason, in addition staphylococcal enterotoxin was detected in SF; simultaneously the bloods of the patients were assayed by the same toxins and compare the results. In this study, the presence of staphylococcal enterotoxin C was identifying in two levels. First, the proteinous enterotoxin state and the second it's encoding gene in both SF and blood was tracing by ELISA and PCR methods respectively. While, several researchers have been discussion the inflammatory effects of microbial metabolites (20,21). Others have tried to show the relationship of microbium and inflammatory disease (22-24). In the present study, Staphylococcal enterotoxin C as a new biomarker was detected in SF and blood of RA patients by two methods. Although, the ELISA was more rapid for detect of enterotoxin C protein in blood. The PCR molecular method had shown more positive results. The reason for the incompatibility of the ELISA and PCR results might have been related to under low sensitivity concentration of the enterotoxin C. While, the PCR have ability to amplified even one molecule of the enterotoxin C encoding gene. Taken together, these results might be most important in related pathophysiology and clinical diagnosis of RA disease.

## REFERENCES


1. Leung, D. Y. Atopic dermatitis and the immune system: the role of superantigens and bacteria. *J. Am. Acad. Dermatol.* 45: S13-S16; 2001.
2. Salgado-Pabon W, Case-Cook LC, Schlievert PM. Molecular analysis of Staphylococcal superantigens. Methods Mol Biol 2014; 1085:169-85. doi: 10.1007/978-1-62703-664-1_10.
3. Schlievert, P. M., and L. C. Case. Molecular analysis of staphylococcal superantigens. *Methods Mol. Biol.* 2007; 391: 113-26 113-126.
4. Wang, X., H. Zhang, M. Xu, Y. Cai, C. Liu, Z. Su, and C. Zhang. Biological characterization of the zinc site coordinating histidine residues of staphylococcal enterotoxin C2. Microbiology 2009; 155: 680-686.
5. Han, S., K. Kim, Y. Song, H. Kim, J. Kwon, Y. H. Lee, C. K. Lee, S. J. Lee, N. Ha, and K. Kim. Auranofin, an immunosuppressive drug, inhibits MHC class I and MHC class II pathways of antigen presentation in dendritic cells. *Arch. Pharm. Res.* 2008; 31: 370-376.

6. Yanaka, S., M. Kudou, Y. Tanaka, T. Sasaki, S. Takemoto, A. Sakata, Y. Hattori, T. Koshi, S. Futaki, K. Tsumoto, and T. Nakashima. Contribution of the flexible loop region to the function of staphylococcal enterotoxin B. Protein Eng Des Sel . 2010; 23:415-421.
7. Wang, S., Y. Li, H. Xiong, and J. Cao. A broad-spectrum inhibitory peptide against staphylococcal enterotoxin superantigen SEA, SEB and SEC. Immunol. Lett 2008; 121:167-172.
8. Ataee RA, Mohoseni-Moghadam Z, Salimzadeh A, Latifi AM, Ataee MH and Alishiri GH. Enzyme-linked immunosorbent assay for detection of staphylococcal enterotoxins in synovial fluid of rheumatoid arthritis pateints. JAPM 2013; 7: 1113-1119.
9. Singh G, Salvador VB, Bagchi A, Tushabe R, Abrudescu A. Rheumatoid arthritis-associated corneal ulceration with superimposed infection by methicillin-resistant Staphylococcus aureus: a complicated type of corneal melt. Am J Case Rep 2014 Nov 27;15:523-5. doi: 10.12659/AJCR.891127.:523-5.
10. Giri U, Thavalathil BC, Varghese R. Vertebral osteomyelitis in an immunosuppressed patient with rheumatoid arthritis. BMJ Case Rep 2014 ;206944. pii: bcr2014206944. doi: 10.1136/bcr-2014-206944.
11. Ataee RA, Ataee MH, Alishiri GH, Esmaeili D. Staphylococcal enterotoxin C in synovial fluid of patients with rheumatoid arthritis. Iran Red Crescent Med J 2014 Oct 5;16(10):e16075.
12. Ataee RA, Kamali M, Karami A, and Ghorbani M. Standardization of molecular method detection of the entC in S. aureus isolated from human infections and determine it's sequence. Iranian Journal of Military Medicine 2012; 14(3): 232-240.
13. Ataee MH, Alishiri GH, Esmaeili D, Eidei A and Ataee RA. Study of Three Pair Primers PCR to Detect SEC Gene in Synovial Fluid of Rheumatoid Arthritis Patients and Comparison with Elisa. Journal of Pure and Applied Microbiology 2014; 8(spl. edn.1): 125-132.
14. Aletaha D, Neogi T, Silman J.A, Funovits J, Felson T.D, Bingham O.C, and et al. 2010 Rheumatoid Arthritis Classification Criteria. Arthritis & Rheumatism 2010;62(9):2569–2581.
15. Sambrook J, Russel DW. Molecular Cloning: a laboratory manual. 3Ed. New York, Coldspring Harbour Laboratory Press 2001; 417- 421.
16. Gerlach, K., C. Tomuschat, R. Finke, M. S. Staege, C. Brutting, J. Brandt, B. Jordan, R. Schwesig, A. Rosemeier, K. S. Delank, M. E. Kornhuber, and A. Emmer. Experimental Arthritis in the Rat Induced by the Superantigen Staphylococcal Enterotoxin A. *Scand. J. Immunol.* 2017; 85: 191-196.
17. Wooley PH, Cingel B. Staphylococcal enterotoxin B increases the severity of type II collagen induced arthritis in mice. Ann Rheum Dis 1995;54:298-304.
18. Ataee, R. A., M. H. Ataee, G. H. Alishiri, and D. Esmaeili. Staphylococcal enterotoxin C in synovial fluid of patients with rheumatoid arthritis. *Iran Red. Crescent. Med. J.* 2014; 16: e16075.
19. Zahiri, Y. S., R. A. Ataee, G. H. Alishiri, and M. Movahedi. Bacteriological and molecular assessment of staphylococcal enterotoxin e in the blood of patients with rheumatoid arthritis. *Jundishapur. J. Microbiol.* 2015; 8: e16621.
20. Nowrouzian, F. L., G. Lina, E. Hodille, E. Lindberg, B. Hesselmar, R. Saalman, I. Adlerberth, and A. E. Wold. Superantigens and adhesins of infant gut commensal Staphylococcus aureus strains and association with subsequent development of atopic eczema. *Br. J. Dermatol.* 2017; 176: 439-445.
21. Abdelnour, A., Y. X. Zhao, T. Bremell, R. Holmdahl, and A. Tarkowski. Role of superantigens in experimental arthritis. *Springer Semin. Immunopathol.* 1996;17: 363-373.
22. Li, J., J. Yang, Y. W. Lu, S. Wu, M. R. Wang, and J. M. Zhu. Possible Role of Staphylococcal Enterotoxin B in the Pathogenesis of Autoimmune Diseases. *Viral Immunol.* 2015;28: 354-359.
23. Dar, S. A., S. Das, S. N. Bhattacharya, V. G. Ramachandran, T. Ahmed, B. D. Banerjee, S. Sonthalia, V. Sood, and A. C. Banerjea. Possible role of superantigens in inducing autoimmunity in pemphigus patients. *J. Dermatol.* 2011;38: 980-987.
24. Sawitzke, A. D., H. H. Mu, and B. C. Cole. Superantigens and autoimmune disease: are they involved? *Curr. Opin. Infect. Dis.* 1999;12: 213-219.

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