TÜBİTAK

Research Article

Turk. J. Vet. Anim. Sci.
2011; 35(3): 177-182
© TÜBİTAK
doi:10.3906/vet-1009-480

The increase of $CD4^+CD25^+$ T cells in the peripheral blood of pigs persistently infected with porcine reproductive and respiratory syndrome virus

Jingwen ZENG, Danting HE, Xin GUO, Xinna GE, Hanchun YANG

Key Laboratory of Zoonosis of the Ministry of Agriculture, College of Veterinary Medicine and State Key Laboratory of Agribiotechnology, China Agricultural University, No. 2 Yuanmingyuan West Road, Beijing 100094 - CHINA

Received: 21.09.2010

Abstract: Regulatory T lymphocytes (T-regs) may be involved in suppressing the host's immunity, which is important for viral clearance, and may therefore contribute to persistent viral infection. However, nothing is known regarding the situation of porcine T-regs during persistent infection with porcine reproductive and respiratory syndrome virus (PRRSV). This study, which used flow cytometry and real-time PCR, determined that the frequencies of $CD4^+CD25^+$ T-cells and porcine Forkhead/winged helix transcription factor (FoxP3) mRNA levels in peripheral blood significantly increased during PRRSV persistence, indicating that $CD4^+CD25^+$ T-regs might play an important role in PRRSV persistent infection. This study contributes to a better understanding of the immunological basis of PRRSV persistence.

Key words: Porcine reproductive and respiratory syndrome virus, PRRSV, persistent infection, $CD4^+CD25^+$ T-regs, FoxP3

Introduction

Porcine reproductive and respiratory syndrome (PRRS) has become one of the most economically important diseases currently affecting the swine industry worldwide (1). The disease, which is characterized by reproductive failures in sows and respiratory disease in pigs of all ages, was first described in the US in 1987 (2). The etiological agent known as porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive-stranded RNA virus of the *Arteriviridae* family in the order *Nidovirales* (3). PRRSV initiates infection in pulmonary alveolar macrophages (PAMs), induces a prolonged viremia, and is able to establish a persistent infection at low levels of presence in pigs (4). A number of studies have indicated that PRRSV infection exhibits persistent characteristics in infected pigs (5-7), as can be seen with other members of the arterivirus group (8). Moreover, PRRSV infection may strongly modulate the host's immune responses and could be responsible for the suppression of host immunity to other pathogens (9). Previous studies have provided a lot of evidence to support the idea that PRRSV elicits immunomodulation and immunosuppression (10,11). The mechanism involved in the persistent infection of PRRSV still is poorly understood, however. Observations by Lamontagne et al. suggested that an impaired $CD2^+CD8^{high}$ cell response in mediastinal lymph nodes (MLN) and tonsils favors viral persistence in these organs among persistently infected pigs (12). A weak cell-mediated immune (CMI) response following PRRSV infection was considered to contribute to prolonged PRRSV infection, suggesting that PRRSV suppresses T-cell

* E-mail: jwzengcau@126.com

recognition of infected macrophages (13). Recent data suggest that the delayed response against GP5 in the early stages of PRRSV infection may contribute to the prolonged acute infection and the establishment of persistence (14).

Regulatory T lymphocytes (T-regs) are subsets of T lymphocytes that can suppress and regulate the host's immune response. Among them, $CD4^+CD25^+$ T-reg expressing transcription factor family member Forkhead/winged helix (FoxP3) are the most abundant in lymphoid tissues and peripheral blood (15). This T-reg activity can have both positive and negative effects; although it can be beneficial to the host due to a suppression of tissue damage mediated by virus-specific effector T-cells during viral infection, the same T-cells simultaneously inhibit the host's immunity, which is important for viral clearance, and thus may contribute to persistent viral infection (16). In recent years, a number of works have appeared documenting the role of T-regs in murine chronic viral infections and persistent virus infections in humans (16). The studies reveal that T-regs play an important role in establishing and/or maintaining persistent viral infection (17,18). The existence of porcine T-regs, and their phenotypic and functional characterization has been recognized recently (19,20). However, the situation of T-regs in vivo in PRRSV-infected pigs is unclear. Therefore, in this study we characterized the dynamics of $CD4^+CD25^+$ T-regs and analyzed the FoxP3 mRNA expression in the peripheral blood of pigs persistently infected with PRRSV. This was done in order to provide new information for better understanding the immunological basis of persistent infection and pathogenesis of PRRSV, as well as to aid in developing new strategies for PRRS control.

Materials and methods

Our study was performed on 10 landrace specific, pathogen-free pigs obtained from the Beijing Center for SPF Swine Breeding and Management at 4 weeks of age. All pigs were further confirmed to be free of PRRSV and porcine circovirus type 2 (PCV2) infections using commercial ELISA kits for PRRSV antibody detection (IDEXX Labs Inc., USA) and PCV2 antibody detection (INGEZIM CIRCOVIRUS IgG/IgM kit, Ingenasa, Spain) and with PCR or RT-PCR for viral nucleic acid detection. Pigs were transported to the animal facilities at China Agricultural University (CAU) 3 days prior to the virus challenge and randomly divided into 2 groups (an infection group and a control group, with 5 pigs per group). After the groups were determined, they were raised in 2 separate isolation rooms with individual ventilation. Each pig in the infection group was inoculated intranasally with 2 mL of $10^{4.5}$ TCID50 PRRSV HB-1/3.9 viruses (North American type) which had been adapted to MARC-145 cells from HB-1(sh)/2002, a low-virulence strain isolated in 2002 (21). Each pig in the control group was mock-inoculated with an equivalent dose of MARC-145 cells culture supernatant. All animal work performed in this study was approved by the Beijing Laboratory Animal Management Office.

All pigs were clinically observed and rectal temperatures were recorded daily until 70 days post-infection (PI). Both serum and EDTA-stabilized blood samples were collected on days 0 (prior to infection), 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 PI.

The specific antibodies against the N protein of PRRSV were detected in serum samples using a commercial IDEXX Herdchek PRRS 2XR ELISA kit (IDEXX Labs, Inc., USA) following the recommended procedures. Serum samples were tested at a 1:40 dilution and the results were expressed as S/P (sample-to-positive ratio) value.

PRRSV RNA was extracted from serum samples using the QIAamp Viral RNA Mini kit (QIAGEN GmbH, Germany) and first strand cDNA was synthesized using AMV reverse transcriptase (Promega, USA). The primer pair 5'-AATGGGTCGTAAACCGTGTCGA-3' and 5'-TGTCGTAGTGGGAGTCGTACTA-3' was designed according to the sequence of PRRSV HB-1/3.9 strain (GenBank Accession no. EU360130) and used to amplify a 312-nt *ORF7* gene fragment. Real-time PCR detection of PRRSV cDNA was performed using a continuous fluorescence detector (ABI 7500, USA) with the SYBR Green Real-time PCR Master Mix (TOYOBO, Osaka, Japan). The protocol was conducted as previously described by Chung et al. (22) with minor modifications. The final reaction volume was 20 μL. The thermal profile was 95 °C for

5 min, followed by 40 cycles at 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 35 s. Viral loads were expressed as the mean logarithm of viral genome copy 4 numbers per milliliter serum.

PBMCs were separated from the whole blood using Ficoll-Hypaque (HY Biological Manufacture Co., Ltd, Tianjing, China) density centrifugation. The frequency of $CD4^+CD25^+$ T-cell subsets in PBMCs and $CD4^+$ T cells was analyzed by FCM using an EPICS ELITE cell sorter (Beckman-Coulter, USA) with fluorescein isothiocyanate (FITC)-conjugated anti-porcine CD4 (4515-02, Southern Biotech, USA) and mouse anti-porcine CD25 purified (APG250, Antigenix, USA) coupled with goat anti-mouse IgG1-Phycoerthrin (PE1070-09S, Southern Biotech) as the secondary antibodies. The gates were specifically set using SpectralRed™ (SPRD)-conjugated anti-porcine CD3ε antibodies (4510-13, Southern Biotech).

The total RNA in PBMC was isolated by using TRIZOL reagent. The cDNA was synthesized with the AMV reverse transcriptase (Promega, USA). The following primers were used to amplify FoxP3 target-gene cDNA: porcine β-actin (forward: 5'-GAGTGGGAGATCATGGGTG-3'; reverse: 5'-AGTTGAAGGTGGTCTTGTGG-3') and FoxP3 (forward: 5'-CCGTGGACGAGTTCGAGTTT-3'; reverse: 5'-ACTTCCACCCCCAGTTTTGG-3'). The quantitative analysis of cDNA amplification was assessed by performing PCR reactions containing 1 μg of cDNA template, 0.5 μM each of the primers, and SYBR Green Real-time PCR Master Mix (TOYOBO, Osaka, Japan) in a total volume of 20 μL. The thermal profile for FoxP3 amplification was 95 °C for 5 min, followed by 40 cycles at 94 °C for 20 s and 60 °C for 1 min; the profile for β-actin amplification was 95 °C for 5 min, followed by 40 cycles at 94 °C for 20 s, 59 °C for 20 s, and 72 °C for 35s. Gene expression was analyzed with a continuous fluorescence detector (ABI 7500, USA). Samples were normalized by dividing the quantity of the FoxP3 gene by the value of the endogenous reference gene (β-actin).

The SPSS version 11.5 for Windows (SPSS, Chicago, IL, USA) was used for statistical analysis of the data. FCM data were analyzed using an unpaired Student's t-test. Differences were considered significant at $P < 0.05$.

Results

All pigs in both the HB-1/3.9-infected group and the control group survived until the experiment termination (day 70 PI). The pigs inoculated with HB-1/3.9 showed no obvious clinical signs although depression, anorexia, and respiratory symptoms were occasionally observed in the PRRSV-infected group when the body temperature of the infected pigs rose (40-41 °C) during the first 3 weeks post-infection (data not shown). N protein-specific antibodies of PRRSV were detected in PRRSV-infected pigs on day 7 PI. All pigs in the infected group seroconverted by day 14 PI and remained seropositive throughout the study (Figure 1A). The pigs in the control group were seronegative to PRRSV during the whole experiment.

The viral load and kinetics in the serum of PRRSV-infected pigs were measured by real-time PCR (Figure 1B). PRRSV nucleic acids could be detected in the serum samples of all the infected pigs from day 3 PI onwards. The viremia lasted until the experiment termination (day 70 PI), indicating the existence of persistent PRRSV infection in the inoculated pigs. PRRSV nucleic acids in serum samples from the control pigs were negative throughout the whole experiment.

The frequencies of $CD4^+CD25^+$ T-cell subsets in PBMCs and $CD4^+$ T-cells of PRRSV-infected pigs increased from day 3 PI onwards in comparison to the control pigs (Table). Statistical analysis showed that that the frequencies of $CD4^+CD25^+$ T-cell subsets both in PBMCs and $CD4^+$ T-cells of PRRSV-infected pigs were significantly higher than those of the control pigs from day 7 to 70 PI ($P < 0.05$), indicating that PRRSV infection resulted in the increase of $CD4^+CD25^+$ T-cell subsets. The real-time PCR data showed that the level of FoxP3 mRNA in PBMCs of PRRSV-infected pigs was significantly higher than that of the control pigs from day 3 to 70 PI (Figure 2).

Discussion

Nowadays, effectively controlling PRRS is becoming one of the most challenging issues for the swine industry. This situation may be due in part to the immune response and persistent infection of PRRSV in pigs, a relationship which is not clearly understood yet. Impaired IFN-γ secreting cells,

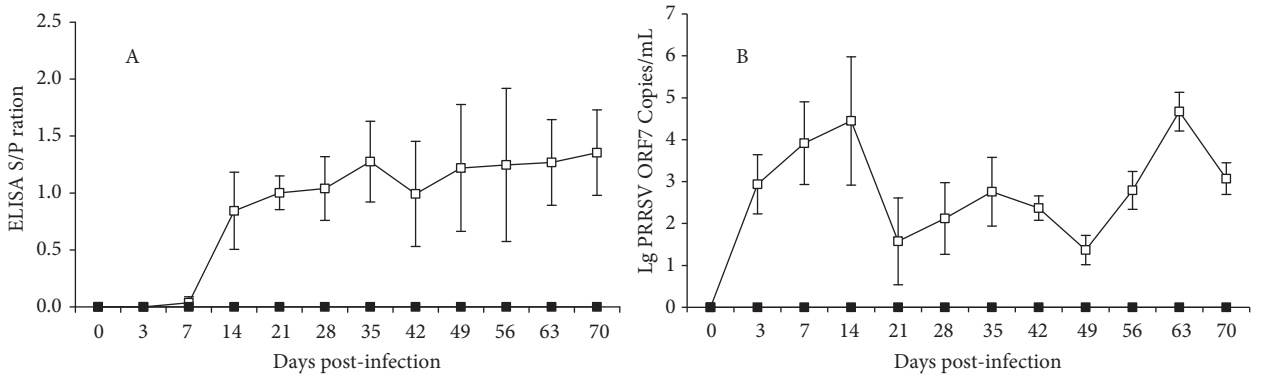

Figure 1. Kinetic of antibody to PRRSV (A) and serum viral loads (B) in the infected (□) and uninfected (■) pigs. The level of antibody was expressed as the mean of sample/positive (S/P) ratio by ELISA. The amount of PRRSV in the serum was determined by real-time PCR and expressed as the mean logarithm of PRRSV ORF7 RNA copy numbers per milliliter. N = 5 in each group; error bars represent standard deviations.

Table. The frequency of $CD4^+CD25^+$ T-cell subsets in peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T-cells as determined by flow cytometry (FCM).

DPI	The frequency of $CD4^+CD25^+$ T-cell subsets in			
	PBMCs (%) of		$CD4^+$ T-cells (%) of	
	Uninfected pigs	Infected pigs	Uninfected pigs	Infected pigs
0	3.50 ± 1.08	4.00 ± 1.19	7.72 ± 1.04	7.84 ± 0.74
3	3.44 ± 1.41	5.24 ± 1.61	7.00 ± 2.23	11.42 ± 4.59
7	3.64 ± 1.30	5.94 ± 0.86*	7.80 ± 1.42	11.60 ± 2.31*
14	3.76 ± 0.73	7.12 ± 2.21*	8.16 ± 1.35	14.84 ± 2.61*
21	3.44 ± 0.68	7.32 ± 0.83*	6.94 ± 1.11	11.50 ± 1.28*
28	3.14 ± 0.64	6.60 ± 2.00*	7.58 ± 0.89	13.38 ± 2.60*
35	3.56 ± 0.47	6.70 ± 1.76*	9.26 ± 0.93	13.48 ± 2.27*
42	3.46 ± 0.59	7.44 ± 1.82*	8.52 ± 0.61	15.72 ± 2.60*
49	3.64 ± 0.99	6.82 ± 1.25*	8.78 ± 0.95	14.46 ± 1.52*
56	3.20 ± 0.49	7.94 ± 1.85*	8.58 ± 0.45	17.42 ± 2.82*
63	3.28 ± 0.81	7.32 ± 1.27*	8.46 ± 1.28	16.52 ± 2.75*
70	3.66 ± 0.84	8.54 ± 1.63*	9.42 ± 1.75	17.40 ± 1.82*

Data were the means ± standard derivations (n = 5 in each group)
* indicates statistical significance as compared to the uninfected pigs ($P < 0.05$)

delayed generation of neutralizing antibody, and imbalanced Th1/Th2 immune responses all appear to contribute to the viral persistence following PRRSV infection (9,11,14,23). It is well known that a controlled balance between initiation and down-regulation of the immune response is vital for

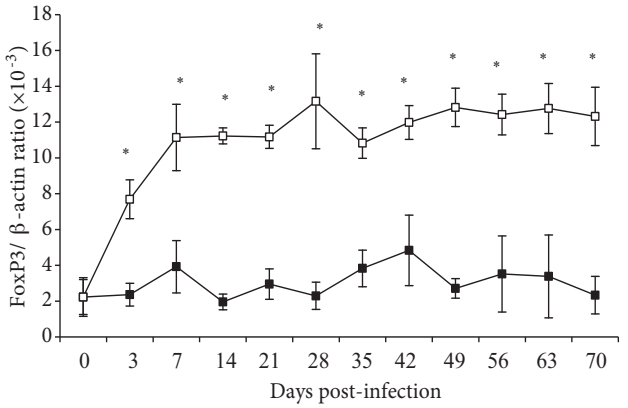

Figure 2. The expression of porcine Forkhead/winged helix transcription factor (FoxP3) mRNA in peripheral blood mononuclear cells (PBMCs) in PRRSV-infected (□) and -uninfected (■) pigs by real-time PCR analysis. Error bars represent standard deviations. * $P < 0.05$.

maintaining immune homeostasis (24). Regulatory T-cells (T-regs) are subsets of T lymphocytes which can suppress and regulate the host's immune response. These cells develop in the thymus and co-express the differentiation antigens CD4 and CD25, which are their prominent phenotypes (19,25). In recent years, the accumulated evidence has revealed that $CD4^+CD25^+$ T-regs play a pathogenic role in several types of persistent viral infections in mice and humans, such as hepatitis B (26), hepatitis C (27), and human immunodeficiency virus (28).

Recently, Käser et al. confirmed the existence of porcine $CD4^+CD25^+$ T-regs in the PBMCs of healthy pigs and characterized their phenotype and suppressive capacities (19). In this study, we used FCM to analyze the frequency of $CD4^+CD25^+$ T-cells and their dynamics in the peripheral blood of pigs persistently infected with PRRSV. Our data showed a significantly higher proportion of $CD4^+CD25^+$ T-cells in the PBMCs and $CD4^+$ T-cells of PRRSV-infected pigs compared with the control pigs (Table), indicating that PRRSV infection induces an increased frequency of $CD4^+CD25^+$ T-cells in pigs. It should be noted that CD25 is an activation marker for T-regs, which also present on the surface of the T helper cells Th1 and Th2 (29). Our results also showed that there were significant increases in FoxP3 mRNA levels in the PBMCs of pigs persistently infected with PRRSV when compared to pigs in the control group between days 3 and 70 PI. Since FoxP3 is considered to be a specific molecular marker of T-reg cells for discrimination between T-reg cells and activated regulatory T-cells (19,29), our results suggest that $CD4^+CD25^+$ T-regs might play an important role in the persistent infection of PRRSV. To our knowledge, this is the first observation regarding the change of $CD4^+CD25^+$ T-regs in the peripheral blood of persistently PRRSV-infected pigs. The increased circulation of $CD4^+CD25^+$ T-cells and FoxP3 mRNA levels observed in this study is in accordance with results from other human viruses which can induce persistent infection (30).

The present study demonstrates that PRRSV infection may result in increases in $CD4^+CD25^+$ T-cells and FoxP3 mRNA expression in the peripheral blood of pigs, implying that $CD4^+CD25^+$ regulatory T lymphocytes might play an important role in the persistent infection of PRRSV. This finding provides new clues to help better our understanding of the immunological basis of persistent infection of PRRSV.

Acknowledgements

This work was supported by the National Natural Science Fund for Distinguished Young Scholars (30825031) from the National Natural Science Foundation of China, National Key Basic Research Plan Grant (#2005CB523204). The authors thank Dr. Yonggang Xu for technical support with the flow cytometry analysis.

References

1. Neumann, E.J., Kliebenstein, J.B., Johnson, C.D., Mabry, J.W., Bush, E.J., Seitzinger, A.H., Green, A.L., Zimmerman, J.J.: Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. J. Am. Vet. Med. Assoc., 2005; 227: 385-392.
2. Rossow, K.D.: Porcine reproductive and respiratory syndrome. Vet. Pathol., 1998; 35: 1-20.
3. Cavanaugh, D.: Nidovirales: a new order comprising coronaviridae and arteriviridae. Arch. Virol., 1997; 142: 629-633.

4. Cho, J.G., Dee, S.A.: Porcine reproductive and respiratory syndrome virus. Theriogenology, 2006; 66: 655-662.
5. Allende, R., Laegreid, W.W., Kutish, G.F., Galeota, J.A., Wills, R.W., Osorio, F.A.: Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. J Virol., 2000; 74: 10834-10837.
6. Batista, L., Pijoan, C., Dee, S., Olin, M., Molitor, T., Joo, H.S., Xiao, Z., Murtaugh, M.: Virological and immunological responses to porcine reproductive and respiratory syndrome virus in a large population of gilts. Can. J. Vet. Res., 2004; 68: 267-273.
7. Wills, R.W., Doster, A.R., Galeota, J.A., Sur, J.H., Osorio, F.A.: Duration of infection and proportion of pigs persistently infected with porcine reproductive and respiratory syndrome virus. J. Clin. Microbiol., 2003; 41: 58-62.
8. Plagemann, P.G., Moennig, V.: Lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus: a new group of positive-strand RNA viruses. Adv. Virus Res., 1992; 41: 99-192.
9. Mateu, E., Diaz, I.: The challenge of PRRS immunology. Vet. J., 2008; 177: 345-351.
10. Drew, T.W.: A review of evidence for immunosuppression due to porcine reproductive and respiratory syndrome virus. Vet. Res., 2000; 31: 27-39.
11. Diaz, I., Darwich, L., Pappaterra, G., Pujols, J., Mateu, E.: Immune responses of pigs after experimental infection with a European strain of porcine reproductive and respiratory syndrome virus. J. Gen. Virol., 2005; 86: 1943-1951.
12. Lamontagne, L., Pagé, C., Larochelle, R., Magar, R.: Porcine reproductive and respiratory syndrome virus persistence in blood, spleen, lymph nodes, and tonsils of experimentally infected pigs depends on the level of $CD8^{high}$ T cells. Viral. Immunol., 2003; 16: 395-406.
13. Xiao, Z., Batista, L., Dee, S., Halbur, P., Murtaugh, M.P.: The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. J. Virol., 2004; 78: 5923-5933.
14. Mulupuri, P., Zimmerman, J.J., Hermann, J., Johnson, C.R., Cano, J.P., Yu, W., Dee, S.A., Murtaugh, M.P.: Antigen-specific B-cell responses to porcine reproductive and respiratory syndrome virus infection. J. Virol., 2008; 82: 358-370.
15. Campbell, D.J., Ziegler, S.F.: FOXP3 modifies the phenotypic and functional properties of regulatory T cells. Nature Rev. Immunol., 2007; 7: 305-310.
16. Li, S., Gowans, E.J., Chougnet, C., Plebanski, M., Dittmer, U.: Natural regulatory T cells and persistent viral infection. J. Virol., 2008; 82: 21-30.
17. Yamano, Y., Takenouchi, N., Li, H.C., Tomaru, U., Yao, K., Grant, C.W., Maric, D.A., Jacobson, S.: Virus-induced dysfunction of $CD4^+CD25^+$ T cells in patients with HTLV-1-associated neuroimmunological disease. J. Clin. Invest., 2005; 115: 1361-1368.
18. Robertson, S.J., Messer, R.J., Carmody, A.B., Hasenkrug, K.J.: In vitro suppression of $CD8^+$ T cell function by Friend virus-induced regulatory T cells. J. Immunol., 2006; 176: 3342-3349.
19. Käser, T., Gerner, W., Hammer, S.E., Patzl, M., Saalmüller, A.: Phenotypic and functional characterization of porcine $CD4+CD25^{high}$ regulatory T cells. Vet. Immunol. Immunopathol., 2008; 122: 153-158.
20. Käser, T., Gerner, W., Hammer, S.E., Patzl, M., Saalmüller, A.: Detection of Foxp3 protein expression in porcine T lymphocytes. Vet. Immunol. Immunopathol., 2008; 125: 92-101.
21. Gao, Z.Q., Guo, X., Yang, H.C.: Genomic characterization of two Chinese isolates of porcine respiratory and reproductive syndrome virus. Arch. Virol., 2004; 149: 1341-1351.
22. Chung, W.B., Chan, W.H., Chaung, H.C., Lien, Y., Wu, C.C., Huang, Y.L.: Real-time PCR for quantitation of porcine reproductive and respiratory syndrome virus and porcine circo-virus type2 in naturally infected and challenged pigs. J. Virol. Methods, 2005; 124: 11-19.
23. Lohse, L., Nielsen, J., Eriksen, L.: Temporary CD8+ T-cell depletion in pigs did not exacerbate infection with porcine reproductive and respiratory syndrome virus (PRRSV). Viral Immunol., 2004; 17: 594-603.
24. Miyara, M., Sakaguchi, S.: Natural regulatory T cells: mechanisms of suppression. Trends Mol. Med., 2007; 13: 108-116.
25. Shevach, E.M.: Regulatory T cells in autoimmunity. Annu. Rev. Immunol., 2000; 18: 423-449.
26. Stoop, J.N., van der Molen, R.G., Baan, C.C., van der Laan, L.J.W., Kuipers, E.J., Kusters, J.G., Janssen, H.L.A.: Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. Hepatol., 2005; 41: 771-778.
27. Cabrera, R., Tu, Z., Xu, Y., Firpi, R., Rosen, H.R., Liu, C., Nelson, D.R.: An immunomodulatory role for $CD4^+CD25^+$ regulatory T lymphocytes in hepatitis C virus infection. Hepatol., 2004; 40: 1062-1071.
28. Kinter, A.L., Hennessey, M., Bell, A., Kern, S., Lin, Y., Daucher, M., Planta, M., McGlaughlin, M., Jackson, R., Ziegler, S.F., Fauci, A.S.: $CD25^+CD4^+$ Regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate $CD4^+$ and $CD8^+$ HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. J. Exp. Med., 2004; 200: 331-343.
29. Andaloussi, A.E., Lesniak, M.S.: An increase in $CD4^+CD25^+FOXP3^+$ regulatory T cells in tumor-infiltrating lymphocytes of human glioblastoma multiforme. Neuro Oncol., 2006; 8: 234-243.
30. Yang, G., Liu, A., Xie, Q., Guo, T.B., Wan, B., Zhou, B., Zhang, J.Z.: Association of CD4+CD25+Foxp3+ regulatory T cells with chronic activity and viral clearance in patients with hepatitis B. Int. Immunol., 2007; 19: 133-140.