

Increased Numbers of CD4⁺CD25⁺ and CD8⁺CD25⁺ T-Cells in Peripheral Blood of Patients with Rheumatoid Arthritis with Parvovirus B19 Infection

MILDA NACIUTE¹, GABRIELE MACIUNAITE¹, DIANA MIELIAUSKAITE¹, RITA RUGIENE^{1,2},
AUKSE ZINKEVICIENE¹, MYKOLAS MAURICAS¹, MODRA MUROVSKA³ and IRUTE GIRKONTAITE¹

¹Department of Immunology, State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania;

²Centre of Rheumatology, Vilnius University, Vilnius, Lithuania;

³A. Kirchenstein Institute of Microbiology and Virology, Riga Stradins University, Riga, Latvia

Abstract. *Aim: To investigate T-cell subpopulations in peripheral blood of human parvovirus B19 DNA-positive (B19⁺) and -negative (B19⁻) patients with rheumatoid arthritis (RA) and healthy persons. Patients and Methods: Blood samples were collected from 115 patients with RA and 47 healthy volunteers; 27 patients with RA and nine controls were B19⁺. Cluster of differentiation (CD) 4, 8, 25 and 45RA were analyzed on blood cells. CD25 expression on CD4⁺CD45RA⁺, CD4⁺CD45RA⁻, CD8⁺CD45RA⁺, CD8⁺CD45RA⁻ subsets were analyzed by flow cytometry. Results: The percentage of CD25^{low} and CD25^{hi} cells was increased on CD4⁺CD45RA⁺, CD4⁺CD45RA⁻ T-cells and the percentage of CD25⁺ cells was increased on CD8⁺CD45RA⁺, CD8⁺CD45RA⁻ T-cells of B19⁺ patients with RA in comparison with B19⁻ patients and controls. Conclusion: Raised levels of CD4 and CD8 regulatory T-cells in B19⁺ RA patients could cause down-regulation of antiviral clearance mechanisms and lead to activation of persistent human parvovirus B19 infection in patients with RA.*

Human parvovirus B19 (B19V) is a small DNA virus with a worldwide distribution. It replicates in red blood cell precursors in the bone marrow; however B19V can also persist in other cells (1, 2). B19V DNA has been found in peripheral blood (PB) and synovial fluid cells of patients

with rheumatoid arthritis (RA) (3, 4). Moreover, the presence of the main B19V capsid protein has been detected in synovial cells, including lymphocytes, macrophages, and neutrophils (5). B19V is associated with many clinical disorders. B19V infection in adults can be asymptomatic or can cause various complications such as arthralgia, chronic arthritis, transient aplastic crisis, and chronic anemia (6, 7). There exists some evidence that B19V infection is more frequent in patients with certain autoimmune diseases, such as RA. It has been shown that the prevalence of B19V DNA in serum, plasma or PB cells is significantly higher in patients with RA than in controls (3, 8-10). RA clinical activity is higher in patients with latent or persistent B19V infection (4). Nevertheless the etiological associations with RA are conflicting and some authors conclude that B19V is not involved in the etiopathogenesis of RA (11, 12).

The aim of this study was to clarify the possible involvement of B19V infection in RA pathogenesis by investigating the T-cell subpopulations in B19⁺ and B19⁻ patients with RA and B19⁺ and B19⁻ healthy volunteers.

Patients and Methods

Patients. Patients with RA and healthy volunteers as the control group were enrolled in this study. Participants were selected from patients seen at the Vilnius University Hospital Santariskiu clinics. B19V genomic DNA was determined in the whole blood and cell-free blood plasma using nested polymerase chain reaction (PCR). The individuals in which B19V DNA was detected (independently in whole blood or cell-free plasma) were designated as B19⁺. Similarly, when B19V DNA was not detected, they were designated as B19⁻. We investigated 115 patients suffering from RA (27 B19⁺ and 88 B19⁻) and 47 (nine B19⁺ and 38 B19⁻) age- and sex-matched healthy volunteers. The mean age of B19⁺ and B19⁻ patients and controls was 58.3±13.6 and 58.3±13.0, and 55.3±9.0 and 49.2±11.3 years, respectively. Six B19⁺ patients with RA and eight B19⁺ controls had persistent infection in latent phase; 19 patients with RA and none of controls had persistent infection in

This article is freely accessible online.

Correspondence to: Irute Girkontaite, State Research Institute Centre for Innovative Medicine, Department of immunology, Santariskiu 5, LT-08409 Vilnius, Lithuania. Tel: +370 61003407, e-mail: igirkontaite@yahoo.com

Key Words: Parvovirus B19, rheumatoid arthritis, Treg, CD4⁺CD25⁺, CD8⁺CD25⁺.

active phase; one patient with RA had acute or persistent infection in active phase; one patient with RA and one control had acute infection. A total of 76 B19⁺ patients with RA and 30 B19⁺ controls had had past infection; 12 B19⁺ patients with RA and eight B19⁺ controls were without infection. A more detailed description of the patients was published early (10). This study was approved by the Vilnius Regional Biomedical Research Ethics Committee (approval number 158200-12-515-157).

Blood samples for peripheral blood mononuclear cell (PBMC) isolation were collected in vacutainers with K₂EDTA and processed within 4 hours of collection. PBMCs were prepared by centrifugation over Lymphoprep (Fresenius Kabi Norge AG, Uppsala, Sweden) gradient, suspended in freezing medium (10% dimethyl sulfoxide, 90% fetal calf serum), frozen and stored in liquid nitrogen until use.

Flow cytometry and antibodies. Frozen PBMCs were thawed and stained with the following antibodies: CD4-fluorescein (FITC), CD8-phycoerythrin (PE), CD25- allophycocyanin (APC) (Biolegend, San Diego, CA, USA) and CD45RA-peridinin chlorophyll protein (PerCP) (MiltenyiBiotec, Bergisch Gladbach, Germany). The expression of cell surface markers was analyzed by four-color flow cytometer FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis. The Mann-Whitney *U*-test was used to compare two groups of patients with non-normal distribution of data. A value of $p < 0.05$ was considered significant.

Results

Increased numbers of CD4⁺CD25⁺ T-cells in the blood of B19⁺ patients with RA. The subsets of T-cells were investigated in PB of B19⁺ and B19⁺ patients with RA in comparison to healthy persons. The percentage of CD4⁺ cells out of all lymphocytes was significantly ($p < 0.05$) higher in the blood of patients with RA in comparison with the controls (Figure 1a).

CD4⁺ T-cells were analyzed further according to the expression of CD45RA and CD25 (Figure 2b). CD45RA⁺ naïve and CD45RA⁺ activated cells were divided according to the expression of CD25: CD25⁺, CD25^{low} and CD25^{hi} (Figure 1c). The percentage of CD25^{low} and CD25^{hi} cells out of CD4⁺CD45RA⁺ cells and out of CD4⁺CD45RA⁺ cells were significantly ($p < 0.005$) higher in PB of B19⁺ patients with RA in comparison with those who were B19⁺ and B19⁺ or B19⁺ controls (Figure 1c). The percentage of CD25⁺CD4⁺ cells (out of both naïve CD45RA⁺ and activated CD45RA⁺ cells) in B19⁺ patients with RA was lower than in other groups.

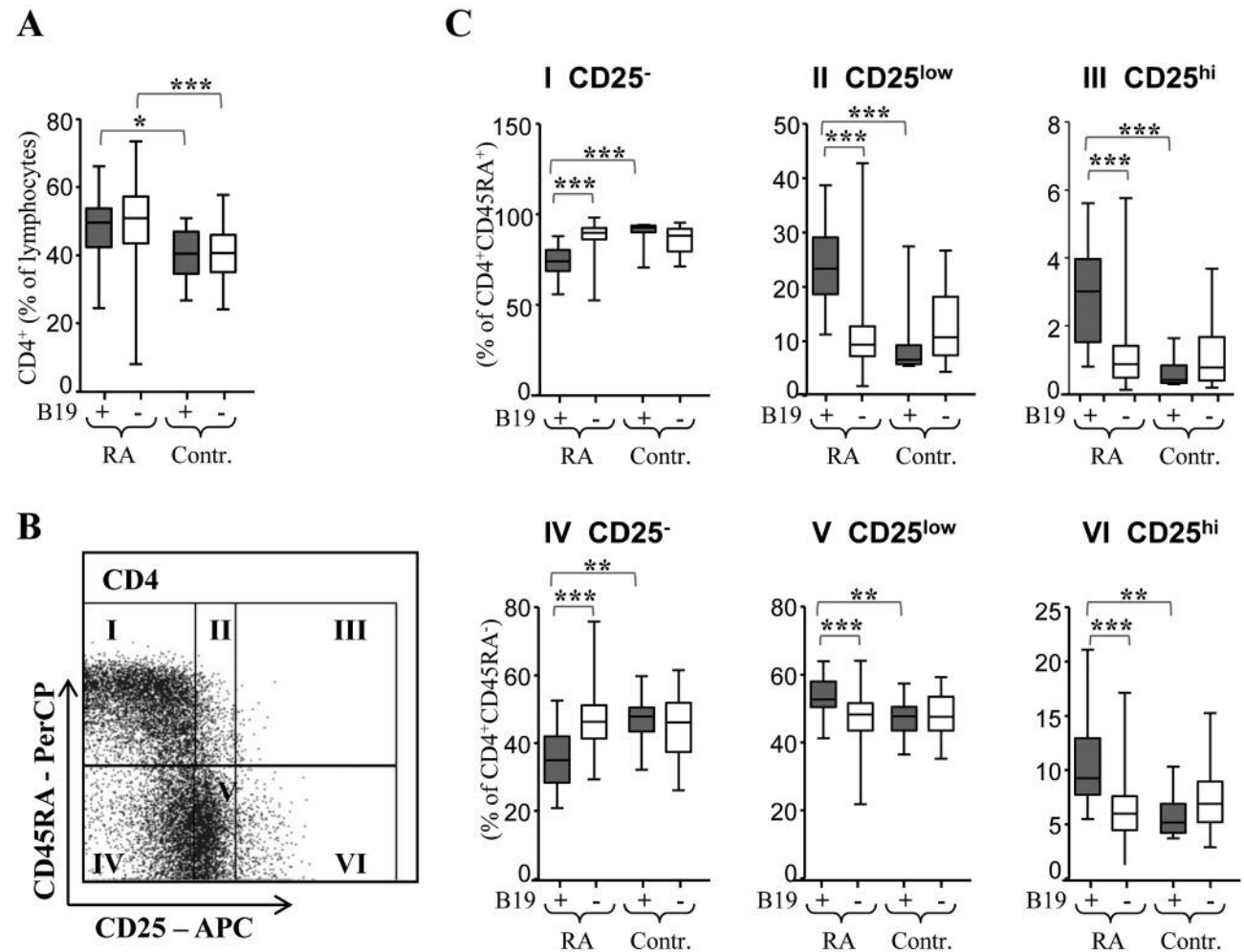
Increased numbers of CD8⁺CD25⁺ T-cells in the blood of B19⁺ patients with RA patients. The percentage of CD8⁺ T-cells from lymphocytes was similar in PB of B19⁺ patients with RA, B19⁺ controls and B19⁺ controls. However B19⁺ patients with RA had a significantly lower percentage of CD8⁺ T-cells. Similar as CD4⁺ T-cells, CD8⁺ T-cells were

analyzed according to their expression of CD25 (CD25⁺ and CD25⁺) in naïve (CD45RA⁺) and activated (CD45RA⁺) subsets (Figure 2b). The percentage of CD25⁺ cells both out of CD8⁺CD45RA⁺ and CD8⁺CD45RA⁺ cells were significantly higher in B19⁺ patients with RA in comparison with those who were B19⁺ and with B19⁺ and B19⁺ controls (Figure 2c). CD25⁺CD8⁺CD45RA⁺ cells accounted for 11% of CD8⁺CD45RA⁺ cells in PB from B19⁺ patients with RA and only for 4-6% in the blood from B19⁺ patients with RA and controls. The percentage of CD25⁺CD8⁺ cells in B19⁺RA patients was significantly lower than that in B19⁺ patients with RA and controls (Figure 2c).

Discussion

Although some evidence argues for a role of B19V in pathogenesis of RA, the knowledge on association between B19V and RA is conflicting. Previously we showed that B19V DNA positivity is higher in patients with RA than in healthy persons (10). Moreover, the majority of B19⁺ patients with RA have persistent infection in active phase, when almost all B19⁺ controls have persistent infection in latent phase.

The T-cell subsets in PB of patients with RA infected with B19V were investigated in this study. Our results show that B19⁺ patients with RA in comparison with those who were B19⁺ and B19⁺ and B19⁺ controls had significantly higher percentages of CD25^{low} and CD25^{hi} T-cells among PB CD4⁺CD45RA⁺ and CD4⁺CD45RA⁺ cells. Likewise, the percentage of CD25⁺ cells was increased in CD8⁺CD45RA⁺ and CD8⁺CD45RA⁺ subsets in B19⁺ patients with RA. According to the literature data, CD4⁺CD45⁺CD25⁺ (populations II and III in Figure 1b) T-cells have low expressing offorkhead box P3 (FOXP3) and are naïve/resting CD4 T-regulatory (suppressive) cells (Tregs) (13, 14). CD4⁺CD45RA⁺CD25^{hi} cells (population VI) are FOXP3^{hi} effector/activated/memory Treg cells with suppressive capability and CD4⁺CD45RA⁺CD25^{low} cells (population V) are FOXP3^{low} cytokine-secreting non-suppressive T-cells (Teffs) (13, 14). CD8⁺CD25⁺ T-cells express FOXP3 and according to the literature data (15) are suppressive. Thus, B19⁺ patients with RA had increased percentages of naïve and memory CD4⁺ and CD8⁺ T-cells. Although the percentage of Teffs was also increased in B19⁺ patients with RA, the ratio of activated CD25^{hi}CD45RA⁺CD4⁺ Tregs and Teffs was higher in B19⁺ patients with RA (data not shown). Van der Geest *et al.* showed that aging disturbs the balance between effector and regulatory CD4⁺ T-cells. It was shown that the Treg/Teff ratio is increased in the memory CD4⁺ T-cell compartment of aged individuals when compared to that of young individuals (16). We did not observe correlation between this ratio and age in the groups of our patients with RA and controls, most likely because there were no very young people in the groups (data not shown).



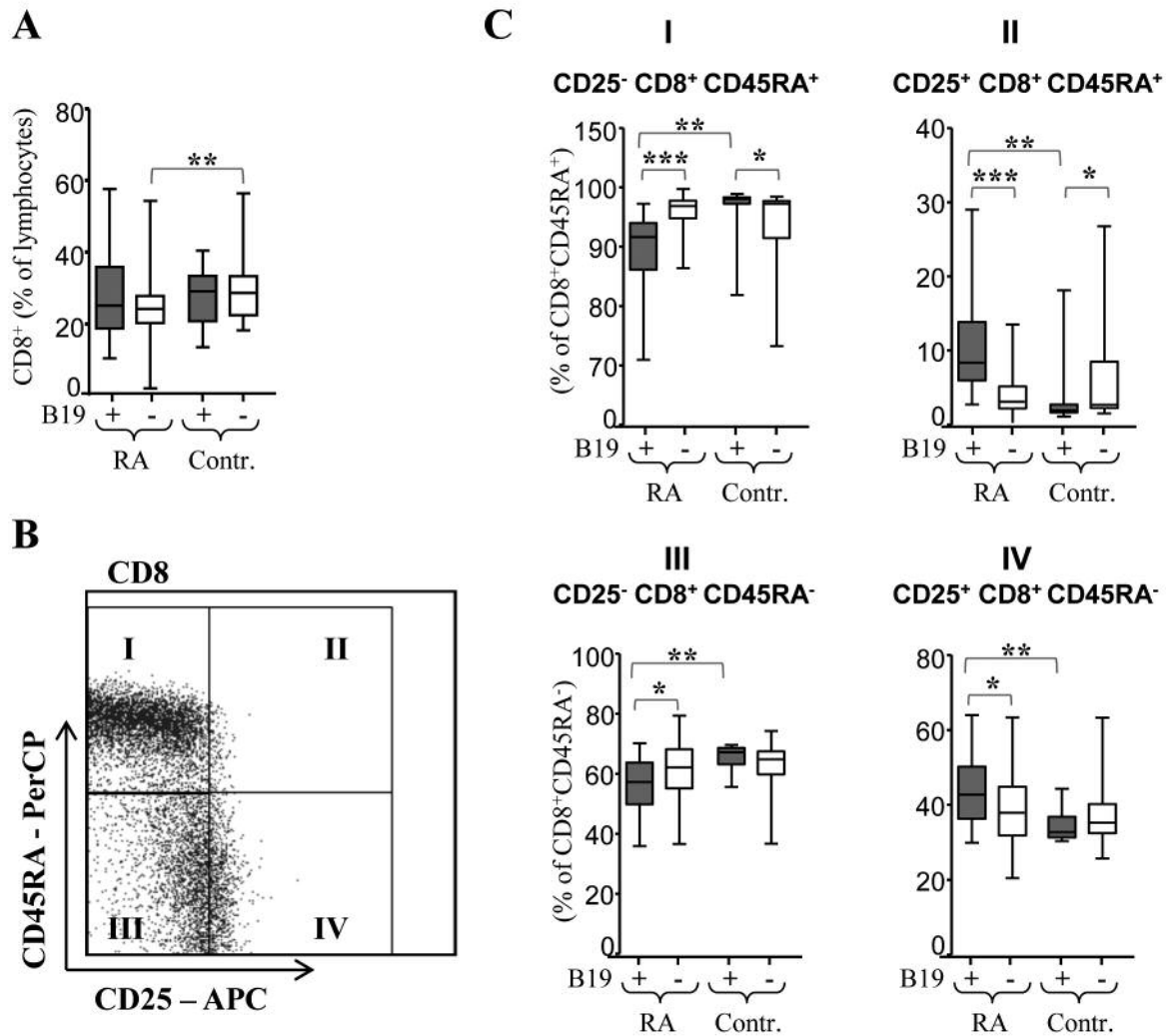


Figure 2. Peripheral blood T-cells from parvovirus B19 (B19) DNA-positive and negative patients with rheumatoid arthritis (RA) and healthy persons (contr.). Peripheral blood mononuclear cells were stained with CD4-fluorescein-, CD8-phycoerythrin-, CD45RA-peridinin chlorophyll protein (PerCP)- and CD25- allophycocyanin (APC)-conjugated antibodies. CD8⁺ cells were gated from CD4/CD8 cell sorting. A: The percentage of CD8⁺ T-cells was calculated from lymphocytes. CD8⁺ T-cells were analyzed further according to CD45RA and CD25 markers. B: Representative dot plot showing the different subpopulations of cells. C: The percentages of subpopulations I and II were calculated from total CD8⁺CD45RA⁺ cells (sum of I and II), the percentages of subpopulations III and IV were calculated from total CD8⁺CD45RA⁻ cells (sum of III and IV). Significantly different at **p*<0.05, ***p*<0.005, and ****p*<0.0005 according to Mann-Whitney U-test.

some viral infections have been shown previously: CD4⁺FOXP3⁺ cells were increased during human T-lymphotropic virus type I (21) and Epstein-Barr virus (22) infections. Patients with acute and chronic hepatitis C (23, 24) or hepatitis B virus infection (25, 26) were found to have a higher CD4⁺CD25⁺ Treg frequency and function in their PB than healthy controls.

In conclusion, an increased percentage of Tregs in B19⁺ patients with RA could contribute to an inadequate immune response against the virus, leading to the activation of persistent infection.

Acknowledgements

This work was supported in part by the Research Council of Lithuania (TAP LLT 02/201), the Taiwan – Latvia – Lithuania Cooperation project 2012-2014.

The Authors thank the patients and healthy volunteers for participating in this study.

References

- Milla F, Feliu E, Ribera JM, Junca J, Flores A, Vidal J, Zarco MA and Masat T: Electron microscopic identification of

- parvovirus virions in erythroid and granulocytic-line cells in a patient with human parvovirus B19 induced pancytopenia. *Leukemia lymphoma* 10: 483-487, 1993.
- 2 Soderlund-Venermo M, Hokynar K, Nieminen J, Rautakorpi H and Hedman K: Persistence of human parvovirus B19 in human tissues. *Pathol Biologie* 50: 307-316, 2002.
 - 3 Kozireva SV, Zestkova JV, Mikazane HJ, Kadisa AL, Kakurina NA, Lejniaks AA, Danilane IN and Murovska MF: Incidence and clinical significance of parvovirus B19 infection in patients with rheumatoid arthritis. *J Rheumatol* 35: 1265-1270, 2008.
 - 4 Kakurina N, Kadisa A, Lejniaks A, Mikazane H, Kozireva S and Murovska M: Use of exploratory factor analysis to ascertain the correlation between the activities of rheumatoid arthritis and infection by human parvovirus B19. *Medicina* 51: 18-24, 2015.
 - 5 Takahashi Y, Murai C, Shibata S, Munakata Y, Ishii T, Ishii K, Saitoh T, Sawai T, Sugamura K and Sasaki T: Human parvovirus B19 as a causative agent for rheumatoid arthritis. *Proc Natl Acad Sci USA* 95: 8227-8232, 1998.
 - 6 Reid DM, Reid TM, Brown T, Rennie JA and Eastmond CJ: Human parvovirus-associated arthritis: a clinical and laboratory description. *Lancet* 1: 422-425, 1985.
 - 7 Lefrere JJ, Courouce AM, Bertrand Y, Girot R and Soulier JP: Human parvovirus and aplastic crisis in chronic hemolytic anemias: a study of 24 observations. *AJH* 23: 271-275, 1986.
 - 8 Chen YS, Chou PH, Li SN, Tsai WC, Lin KH, Tsai KB, Yen JH and Liu HW: Parvovirus B19 infection in patients with rheumatoid arthritis in Taiwan. *J Rheumatol* 33: 887-891, 2006.
 - 9 Caliskan R, Masatlioglu S, Aslan M, Altun S, Saribas S, Ergin S, Uckan E, Koksall V, Oz V, Altas K, Fresko I and Kocazeybek B: The relationship between arthritis and human parvovirus B19 infection. *Rheumatol Int* 26: 7-11, 2005.
 - 10 Naciute M, Mieliauskaite D, Rugiene R, Nikitenkiene R, Jancoriene L, Mauricas M, Nora-Krukke Z, Murovska M and Girkontaite I: The frequency and significance of the parvovirus B19 infection in patients with rheumatoid arthritis. *J Gen Virol* 97: 3302-3312, 2016.
 - 11 Nikkari S, Roivainen A, Hannonen P, Mottonen T, Luukkainen R, Yli-Jama T and Toivanen P: Persistence of parvovirus B19 in synovial fluid and bone marrow. *Ann Rheum Dis* 54: 597-600, 1995.
 - 12 Kerr JR, Cartron JP, Curran MD, Moore JE, Elliott JR and Mollan RA: A study of the role of parvovirus B19 in rheumatoid arthritis. *Br J Rheumatol* 34: 809-813, 1995.
 - 13 Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, Mathian A, Nakahata T, Yamaguchi T, Nomura T, Ono M, Amoura Z, Gorochoy G and Sakaguchi S: Functional delineation and differentiation dynamics of human CD4⁺ T-cells expressing the FoxP3 transcription factor. *Immunity* 30: 899-911, 2009.
 - 14 Seddiki N, Santner-Nanan B, Tangye SG, Alexander SI, Solomon M, Lee S, Nanan R and Fazekas de Saint Groth B: Persistence of naive CD45RA⁺ regulatory T-cells in adult life. *Blood* 107: 2830-2838, 2006.
 - 15 Jebbawi F, Fayyad-Kazan H, Merimi M, Lewalle P, Verougstraete JC, Leo O, Romero P, Burny A, Badran B, Martiat P and Rouas R: A microRNA profile of human CD8(+) regulatory T-cells and characterization of the effects of microRNAs on Treg cell-associated genes. *J Transl Med* 12: 218, 2014.
 - 16 van der Geest KS, Abdulahad WH, Tete SM, Lorencetti PG, Horst G, Bos NA, Kroesen BJ, Brouwer E and Boots AM: Aging disturbs the balance between effector and regulatory CD4⁺ T-cells. *Exp Gerontol* 60: 190-196, 2014.
 - 17 Sempere-Ortells JM, Perez-Garcia V, Marin-Alberca G, Peris-Pertusa A, Benito JM, Marco FM, Zubcoff JJ and Navarro-Blasco FJ: Quantification and phenotype of regulatory T-cells in rheumatoid arthritis according to disease activity score-28. *Autoimmunity* 42: 636-645, 2009.
 - 18 Zare HR, Habibagahi M, Vahdati A and Habibagahi Z: Patients with active rheumatoid arthritis have lower frequency of nTregs in peripheral blood. *Iran J Immunol* 12: 166-175, 2015.
 - 19 Walter GJ, Fleskens V, Frederiksen KS, Rajasekhar M, Menon B, Gerwien JG, Evans HG and Taams LS: Phenotypic, functional and gene expression profiling of peripheral CD45RA⁺ and CD45RO⁺ CD4⁺CD25⁺CD127(low) Treg cells in patients with chronic rheumatoid arthritis. *Arthritis Rheumatol* 68: 103-116, 2016.
 - 20 Kawashiri SY, Kawakami A, Okada A, Koga T, Tamai M, Yamasaki S, Nakamura H, Origuchi T, Ida H and Eguchi K: CD4⁺CD25⁺(high)CD127(low/-) Treg cell frequency from peripheral blood correlates with disease activity in patients with rheumatoid arthritis. *J Rheumatol* 38: 2517-2521, 2011.
 - 21 Barros N, Woll F, Watanabe L and Montes M: Are increased FOXP3⁺ regulatory T-cells responsible for immunosuppression during HTLV-1 infection? Case reports and review of the literature. *BMJ Case Rep* 27: 2012, 2012.
 - 22 Ateyah ME, Hashem ME and Abdelsalam M: Epstein-Barr virus and regulatory T-cells in Egyptian paediatric patients with acute B lymphoblastic leukaemia. *J Clin Pathol* 70: 120-125, 2017.
 - 23 Chalupa P, Davidova A, Beran O, Arientova S, Bostik P, Kapla J, Kondelkova K, Plisek S and Holub M: Effect of antiviral treatment of chronic hepatitis C on the frequency of regulatory T-cells, T-cell activation and serum levels of TGF-beta. *APMIS* 124: 711-718, 2016.
 - 24 Zhai N, Chi X, Li T, Song H, Li H, Jin X, Crispe IN, Su L, Niu J and Tu Z: Hepatitis C virus core protein triggers expansion and activation of CD4⁺CD25⁺ regulatory T-cells in chronic hepatitis C patients. *Cell Mol Immunol* 12: 743-749, 2015.
 - 25 Franzese O, Kennedy PT, Gehring AJ, Gotto J, Williams R, Maini MK and Bertolotti A: Modulation of the CD8⁺-T-cell response by CD4⁺ CD25⁺ regulatory T-cells in patients with hepatitis B virus infection. *J Virol* 79: 3322-3328, 2005.
 - 26 Stoop JN, van der Molen RG, Baan CC, van der Laan LJ, Kuipers EJ, Kusters JG and Janssen HL: Regulatory T-cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. *Hepatology* 41: 771-778, 2005.

Received November 29, 2016

Revised January 24, 2017

Accepted January 27, 2017