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

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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

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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 K_2EDTA 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).

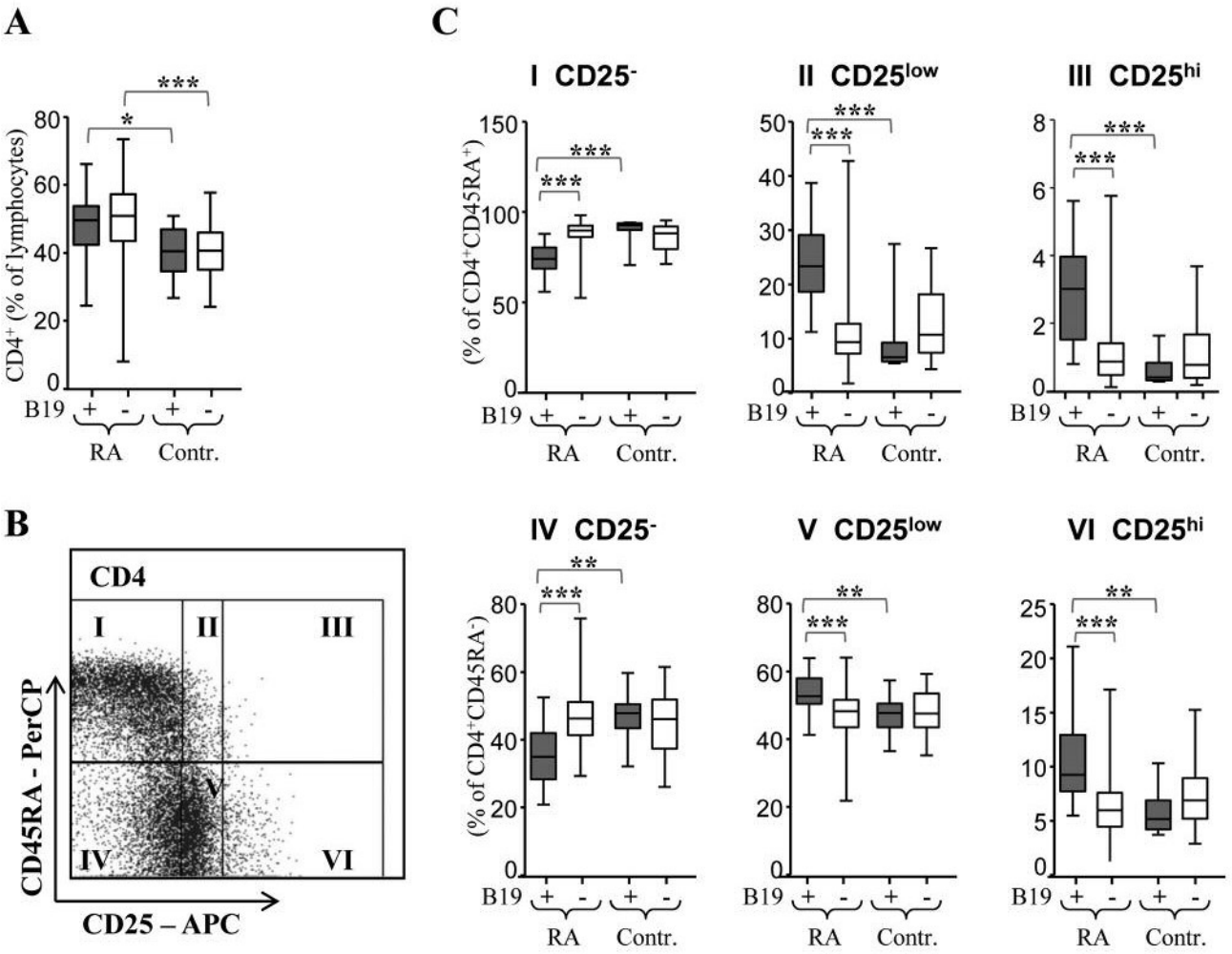

Figure 1. *Peripheral blood partioning of T-cells from parvovirus B19 (B19) DNA-positive and -negative patients with rheumatoid arthritis (RA) and healthy persons (contr.) by immunolabeling. Peripheral blood mononuclear cells were stained with CD4- fluorescein-, CD8-phycoerythrin-, CD45RA-peridinin chlorophyll protein (PerCP)- and CD25-allophycocyanin (APC)-conjugated antibodies. $CD4^+$ cells were gated from CD4/CD8 cell sorting. A: The percentage of $CD4^+$ T-cells was calculated from lymphocytes. $CD4^+$ 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, II and III were calculated from total $CD4^+CD45RA^+$ (sum of I, II and III), the percentages of subpopulations IV, V and VI were calculated from total $CD4^+CD45RA^-$ (sum of IV, V and VI). Significantly different at $^{**}p<0.005$, and $^{***}p<0.0005$ according to Mann-Whitney U-test.*

Tregs play crucial roles in autoimmune diseases, but we did not observe significant differences in the percentage of Tregs in $B19^-$ patients with RA and controls. Literature data show that $CD4^+CD25^+$ Treg counts in patients with RA vary from study to study: compared to the healthy controls, the percentage of $CD4^+$ Tregs in PB from patients with RA was found to be decreased (17, 18), not different (19) or to be reduced in the active stage of RA but not in remission (20).

Eight out of nine $B19^+$ controls (88.9%) had persistent B19V infection in latent phase, when six out of 27 (22.2%) of $B19^+$ patients with RA have persistent infection in latent phase and 19 out of 27 (70.4%) had persistent infection in active phase. Nevertheless $B19^+$RA patients independently on infection activity phase have increased $CD25^{low}$ and $CD25^{hi}$ subpopulations (data not shown). $CD4^+CD25^+$ and $CD8^+CD25^+$ regulatory T-cells regulate immune responses to pathogens. Increased numbers of naïve and activated Tregs in $B19^+$ patients with RA can cause an inability to properly control the host inflammatory response and can lead to T-cell exhaustion, and viral persistence. Increased numbers of Tregs during

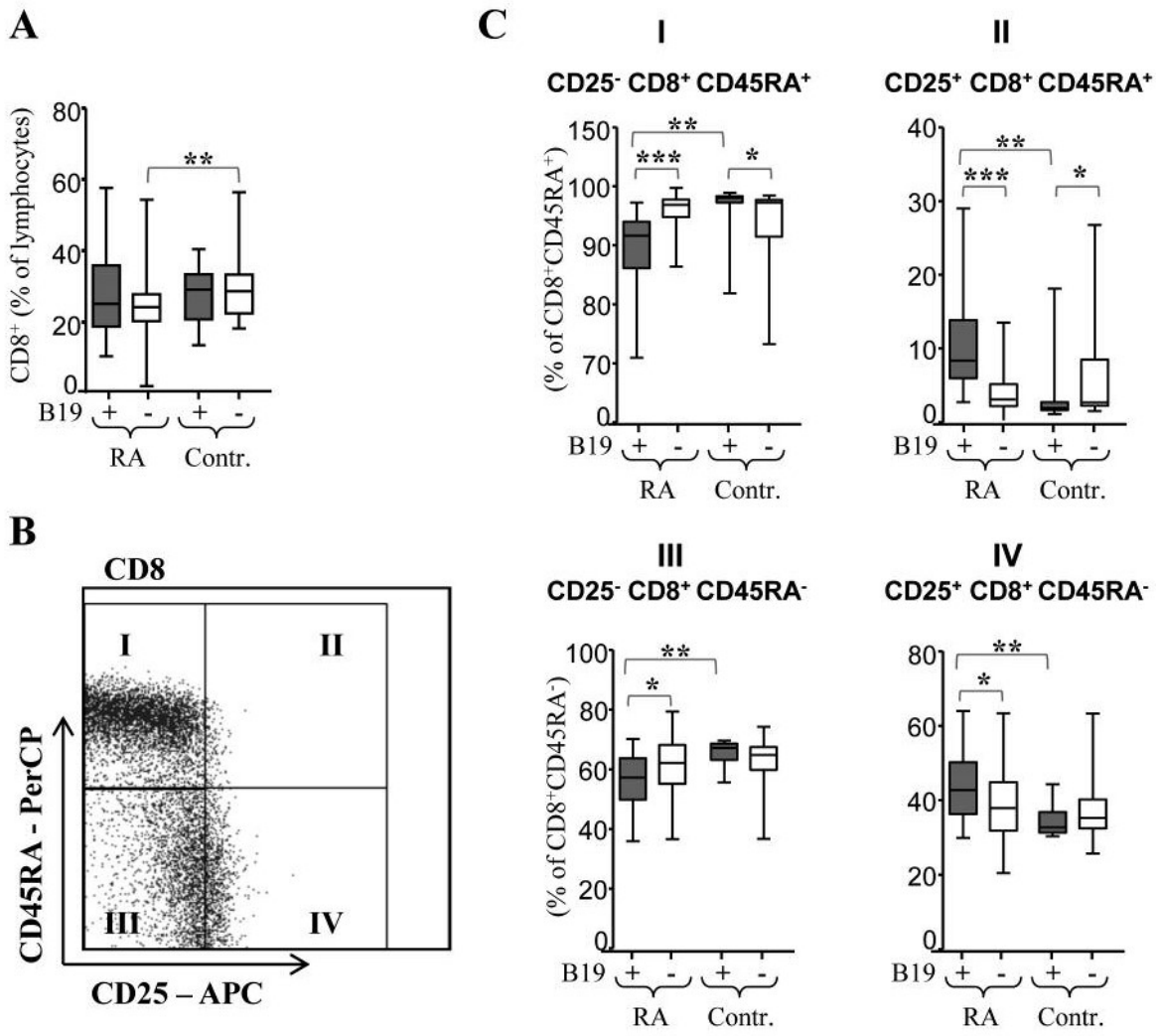

Figure 2. *Peripheral blood T-cells from parvovirus B19 (B19) DNA-positive and negative patients with rheumatoid arthritis (RA) and healthy persons (contr.). Peripheral blood mononuclear cells were stained with CD4-fluorescein-, CD8-phycoerythrin-, CD45RA-peridinin chlorophyll protein (PerCP)- and CD25- 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 $CD4^+CD45RA^-$ cells (sum of IV 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.

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