IL-7 and IL-15 combined with strong TCR stimulation decrease Treg suppressive activity in healthy donors and patients with rheumatoid arthritis

Category: Article

Short title: IL-7 and IL-15 reduce Treg function

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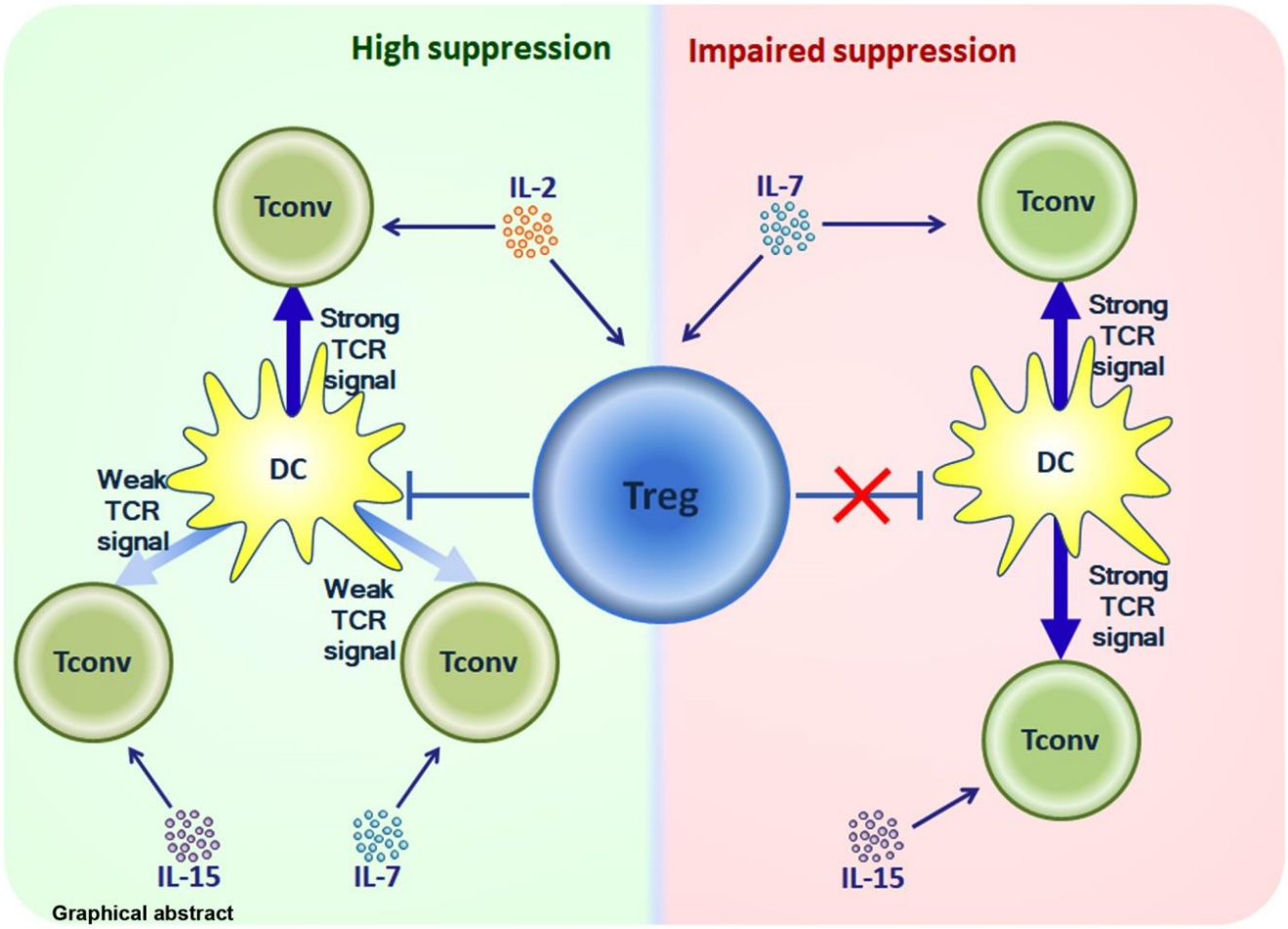


Graphical abstract

Abstract

Objective. Homeostatic proliferation (HP) is a physiological process to reconstitute the T-cell pool after lymphopenia with IL-7 and IL-15 being the key cytokines regulating the process. However, there is no evidence whether these cytokines influence the function of regulatory T cells (Tregs). Since lymphopenia often accompanies autoimmune diseases, we decided to study the proliferation rate and function of Tregs stimulated by IL-7 and IL-15 in patients with rheumatoid arthritis compared to healthy donors.

Materials and Methods. The study used peripheral blood from 14 patients with rheumatoid arthritis and 18 healthy volunteers. Proliferation of purified $CD3^+CD4^+CD25^+CD127^{lo}$ cells was assessed by flow cytometry using CFSE. Tregs were stimulated by anti-CD3, IL-7, IL-15, IL-7 or IL-15 combined with anti-CD3, and by IL-2+anti-CD3, and their functional activity was evaluated in each case by $CD4^+$ and $CD8^+$ cells proliferation inhibition.

Results. The suppressive activity of total pool of Tregs did not differ between rheumatoid arthritis and healthy donors; however, it significantly decreased when IL-7 or IL-15 were applied together with strong TCR stimulation with anti-CD3 antibodies. Herewith Treg proliferation caused by IL-7 and IL-15 was lower in patients than in healthy individuals.

Conclusion. The revealed decrease in Treg suppressive activity can have an impact on the TCR landscape during lymphopenia and lead to the proliferation of potentially self-reactive T-cell clones, which are able to receive relatively strong TCR signals. This may be another explanation why lymphopenia is associated with the development of autoimmune diseases. The revealed decrease of Treg proliferation under IL-7 and IL-15 may lead to a delay in Treg pool reconstitution in patients with rheumatoid arthritis.

Key words: Treg, rheumatoid arthritis, homeostatic proliferation, Treg function, index of suppression.

Introduction

In the course of life, a human body frequently exposes to different stresses, infections, chemicals, radiation, and other physical agents that can potentially lead to the development of lymphopenia. It is well known that an increased risk of autoimmune diseases including rheumatoid arthritis is associated with lymphopenia, which can play a crucial role in the early stages of the disease development [1]. The principal physiological mechanism to restore T-cells pool after lymphopenia is homeostatic proliferation (HP) [2] it being the main source of T-cells after the thymus involution in adulthood [3]. It is well known that the T-cell receptor (TCR) signal, co-stimulation signals, as well as IL-7 and IL-15, are the main factors that provide HP [4-8]. Various cell types produce IL-7 and IL-15, primarily in lymphoid tissues in lymphopenia [9-12]. Both IL-7 and IL-15 belong to the γc-common family and bind with receptors that contain γ-chain (IL-2R-γ) – CD127 and CD215, respectively. Usually, the effect of IL-7 and IL-15 is mediated by the activation of three signal pathways: JAK-STAT, PI3K-Akt, and RAS-RAF-MAPK [13, 14]. TCR signal and the activation of the mentioned signal pathways by IL-7 and IL-15 provide the required level of activation for the survival and proliferation of T-lymphocytes during HP. However, HP can be conventionally divided into a fast and slow depending on its intensity, which directly depends on the TCR signal strength determines by the avidity of the interaction between TCR and peptide in the Major Histocompatibility Complex (pMHC) [15]. Weak or strong TCR signals generate different Akt proteoforms, which determine the activation of different intracellular signaling pathways and thus affect cell fate [16]. Therefore, division of HP into slow and fast has a materialistic background.

For slow HP, it is enough to have a “tonic” TCR signal and elevated levels of IL-7 and IL-15. It has a polyclonal character and, probably, does not lead to negative consequences by maintaining the diversity of TCR. At the same time, fast HP primarily depends on a strong TCR signal, has oligoclonal character, and leads to the changes in the TCR landscape and formation of effector and memory-like T-cells, increasing the risk of tissue inflammation and autoimmune disease development [17-19]. Since the avidity of the TCR-pMHC interaction determines the ability of T cells to compete for proliferation factors in lymphopenic conditions, then only 30% of the total pool of T-lymphocytes, characterized by the highest TCR-pMHC affinity, enters HP in vivo. Since not all possible antigens are present in the body simultaneously and the majority of antigens being self-antigens, potentially self-reactive T-cell clones are selected during HP, changing the overall T-cell landscape [6, 18-21].

Tregs are the main cells to provide tolerance to self-antigens by a wide range of mechanisms. It has been shown that Tregs are able to inhibit T-cell proliferation and cytokine production, thus preventing autoimmunity [22]. However, the role of these cells in the suppression of self-reactive T-cell clones during homeostatic proliferation is understudied. It is assumed that a decreased Treg quantity and their impaired function may lead to the development of autoimmune diseases [23]. At the same time, the existing research results on Treg cells function in patients with rheumatoid arthritis (RA) remain controversial. While some studies demonstrate a decrease in the Treg number or suppressive activity in RA [24-28], others show their preserved functional activity and quantity [29-32]. Nevertheless, despite the increased synovial infiltration of Tregs in RA [33], the inflammation does not resolve, raising the question whether their suppressive activity has actually remained preserved. Thus, this study is aimed to determine the functional activity of Tregs in RA patients and to investigate the influence of humoral factors of HP on Tregs in vitro. For that purpose, we investigated the capacity of Tregs to inhibit the proliferation of $CD4^+$ and $CD8^+$ cells stimulated by various HP

humoral factors. We used IL-7 or IL-15 alone and in combination with anti-CD3 (analog of slow and fast HP, respectively). Since TCR signal and IL-2 are the most important factors for maintenance of Tregs function, we also stimulated the proliferation with anti-CD3+IL-2 combination. Thus, we used the following stimulation conditions: anti-CD3 only, anti-CD3+IL-2, IL-7 only, anti-CD3+IL-7, IL-15 only, anti-CD3+IL-15, also Treg pretreated with IL-7 stimulated by anti-CD3, and control without stimulation.

Materials and Methods

Participants

Eighteen healthy donors (HD) and fourteen patients with RA (according to the ACR/EULAR 2010 criteria) were enrolled in this study conducted at the Immunopathology Hospital Rheumatology Department of the Research Institute for Fundamental and Clinical Immunology, Novosibirsk, Russia. The Local Ethics Committee in accordance with the Declaration of Helsinki (Minutes № 110, October 11, 2018) approved the study. In all cases, the blood was sampled upon written voluntary informed consent. The study included patients with polyarticular form of rheumatoid arthritis with a medium and high disease activity treated from the disease exacerbation at the Research Institute Rheumatology Department. The disease activity was evaluated using DAS-28 score, which was, on average, 5.2±1.6. The average duration of the disease was 5.5±2.7 years (Tab. 1). All patients received disease-modifying antirheumatic drugs (DMARDs), i.e. methotrexate or sulfasalazine as monotherapy or combined with glucocorticoids. Exclusion criteria included an acute or active infection, lactation or pregnancy, biological DMARD or targeted therapy application and a history of cancer or immune deficiency also other immune-related diseases (e.g. type I diabetes, chronic kidney or liver disease etc.). There were no significant differences in sex or average age between HD and RA patients.

Parameter	**Healthy donors**	**RA patients**
Male	6	3
Female	12	11
Age	56,4±11	59,5±11
DAS-28	-	5,2±1,6
ESR (mm/h) Mean ± SD	-	35,4±9,4
CRP (mg/dl) Mean ± SD	-	19,9±6,7
TJC Mean ± SD out of 28	-	7,3±3,2
SJC Mean ± SD out of 28	-	4,2±2,9
Duration of disease	-	5,5±2,7

Table 1. Evaluated clinical parameters. DAS – disease activity score, ESR – erythrocytes sedimentation rate, CRP – C-reactive protein, TJC – tender joint count, SJC – swollen joint count, SD – standard deviation

Cell sorting and Treg suppressive activity assay

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation. Treg cells were purified by immunomagnetic separation by $CD3^{+}CD4^{+}CD25^{+}CD127^{low}$ phenotype using a Miltenyi Biotec MACS Treg Isolation Kit (according to the manufacturer's protocol). The purity of magnetic separation was 94.2 ± 3% (Mean ± SD). The functional activity of Tregs was evaluated by inhibition of $CD4^{+}$ and $CD8^{+}$ lymphocytes proliferation with Treg: PBMC

autologous co-cultures ratios equalling 1:1 (30 000 Treg: 30 000 PBMC). Before cultivation, PBMCs were stained with CFSE (Invitrogen, USA) to assess proliferation. Cells were cultivated over 4 days in 96-well u-bottom plates with RPMI-1640 medium supplemented with 10% FCS and antibiotics the total volume equalling 200µl. The following concentrations of cytokines were applied: anti-CD3 (Sorbent, Russia) – 1 µg/ml, IL-7 (MyBiosource, USA) – 50 ng/ml, IL-15 (MyBiosource, USA) – 50 ng/ml, IL-2 (NPK BIOTECH, Russia) – 100 IU/ml. We used the following stimulation conditions: by anti-CD3, by IL-7, by IL-15, by anti-CD3+IL-2, by anti-CD3+IL-7, and by anti-CD3+IL-15. To directly measure IL-7 effects, Tregs were previously incubated with IL-7 (50 ng/ml) over 2 hours, washed twice, and then cultivated with PBMC stimulated by anti-CD3. CFSE-stained PBMCs were cultured under the same conditions without Treg cells (60 000 per well) to calculate the suppression index (SI). Cells were also cultivated without any stimulation applied to provide the control of cell proliferation. The Treg suppression index was calculated for $CD4^+$ and $CD8^+$ cells by the following formula [34]:

$$\boldsymbol{I = 100 \times (1 - \frac{\%\, Tconv\ proliferation\ with\ Treg}{\%\ of\ Tconv\ proliferation\ in\ the\ absence\ of\ Treg})}$$

CFSE-labeled Tregs with autologous PBMC (30 000: 30 000) were also cultivated with IL-7, IL-15, and anti-CD3+IL-2 stimulation to assess their proliferation. Supernatants were sampled on Day 3 (according to the manufacturer's instructions) to determine ELISA IL-10 (Vector-Best, Russia) and TGFβ (Legend Max, USA) concentrations as main Treg suppression cytokines. CTLA-4, PD-L1, CCR4, and HLA-DR expression on Tregs was estimated by flow cytometry before cultivation and on Day 4 of the experiment (Fig. 1).

In this study we decided to cultivate Tregs without prior activation to measure the initial Tregs functionality and to bring the experiment as close to physiological conditions as possible. The optimal Treg: PBMC ratio was previously determined in experiments as 1:1 corresponding to the existing research results on the topic [32, 35, 36].

Flow cytometry

Phenotype of Treg cells from peripheral blood was tested by the following BioLegend monoclonal antibodies: CD3 by FITC (or PE/Cy7 in the experiments to assess the suppressor index), CD4 – by APC/Cy7, CD25 – by PE, CD127 – by PerCP/Cy5.5, CTLA-4 – by APC, PD-L1 – by APC, HLA-DR – by APC, and CCR4 – by PE/Cy7. Tregs were identified as $CD4^+CFSE^+$ when their phenotypic traits assessed in the cultures (CD4 – by APC/Cy7) (Fig 1, 9). FMO controls were applied for the analysis. Minimum 5000 cells were acquired for the each flow cytometry analysis. BD FACS Canto II cytometer was used for the flow cytometry analysis. All previous steps (PBMCs isolation, Treg sorting, cytometry analysis, and initiation of cultivation) were performed on the day of blood collection without fixing or freezing the cells.

Statistical Analysis

The mean, median, standard deviation (SD) and interquartile range (IQR) were calculated. Shapiro-Wilk test was used to test the normal distribution. The unpaired Student's t-test or Mann-Whitney test was used to compare HC and RA. ANOVA (the Freidman test in case of non-parametric distribution) and post hoc analysis (Tukey and Dunn's multiple comparison tests for parametric and

non-parametric distribution, respectively) were used to compare multiple dependent groups. GraphPad Prism 7.03 was used to perform the statistical analysis.

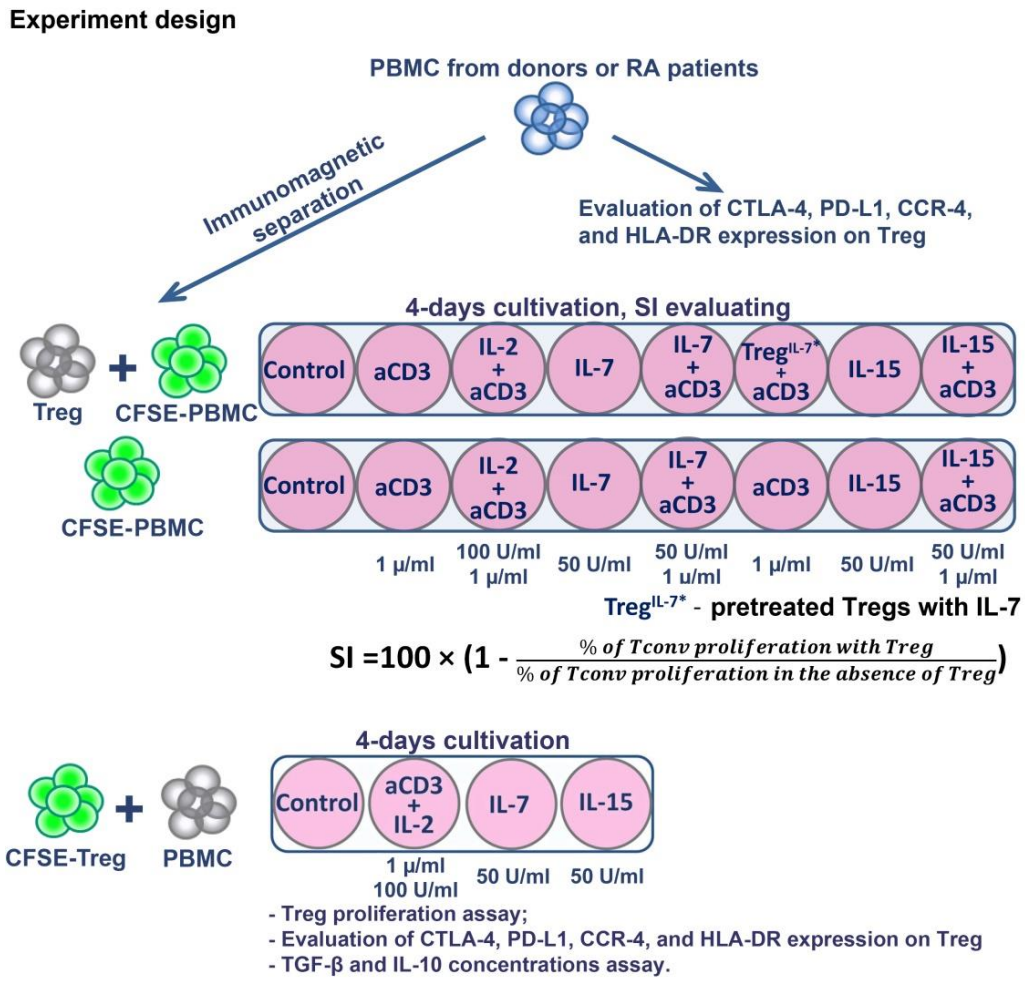


Fig.1. Experiment design.

Results

Strong TCR signal in combination with the influence of HP cytokines reduces suppressive activity of Treg

After 4 days of cultivation, we assessed Tregs suppressive activity under the conditions described above to find that anti-CD3, IL-7, IL-15, and anti-CD3+IL-2 stimulation had similar effect on the Treg suppressive activity for $CD4^+$ cells in healthy donors and RA patients. However, when we imitated the conditions of fast HP and exposed the cells to IL-7 or IL-15 accompanied by strong [16] TCR stimulation (i.e. by anti-CD3+IL-7 or anti-CD3+IL-15) Tregs suppressive activity was significantly reduced as the suppression index for $CD4^+$ was significantly lower in comparison with a separate anti-CD3, anti-CD3+IL2, IL-7, and IL-15 stimulation. The similar trend was observed when Tregs were pretreated with IL-7 (Fig 2).

Similar results were obtained for $CD8^+$ cells. A high SI was observed when we applied IL-7 or IL-15 alone, anti-CD3, or anti-CD3+IL2. In contrast, when IL-7 or IL-15 were combined with anti-CD3, or IL-7 was used for Tregs pretreatment, the SI was significantly reduced (Fig 3).

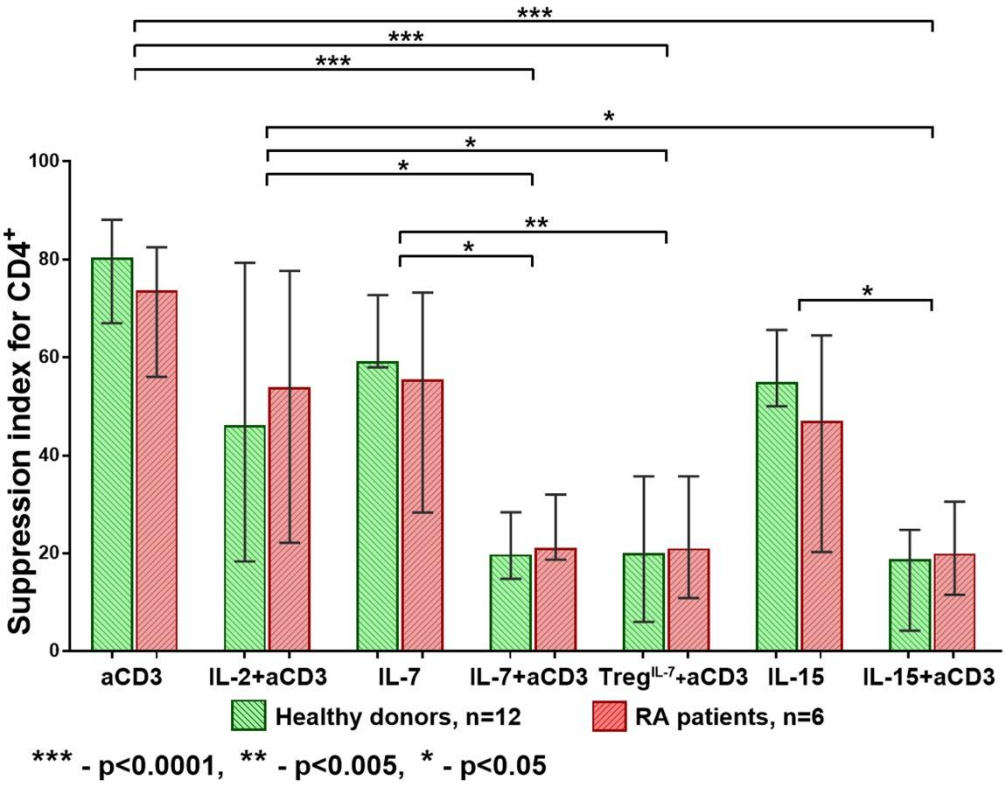


Fig.2. Treg suppression index for CD4$^+$cells. Median, IQR. Comparison of related groups was performed using the Friedman test (ANOVA); post hoc analysis was performed using the Dunn test. Unrelated groups were compared using the Mann-Whitney test; RA – rheumatoid arthritis.

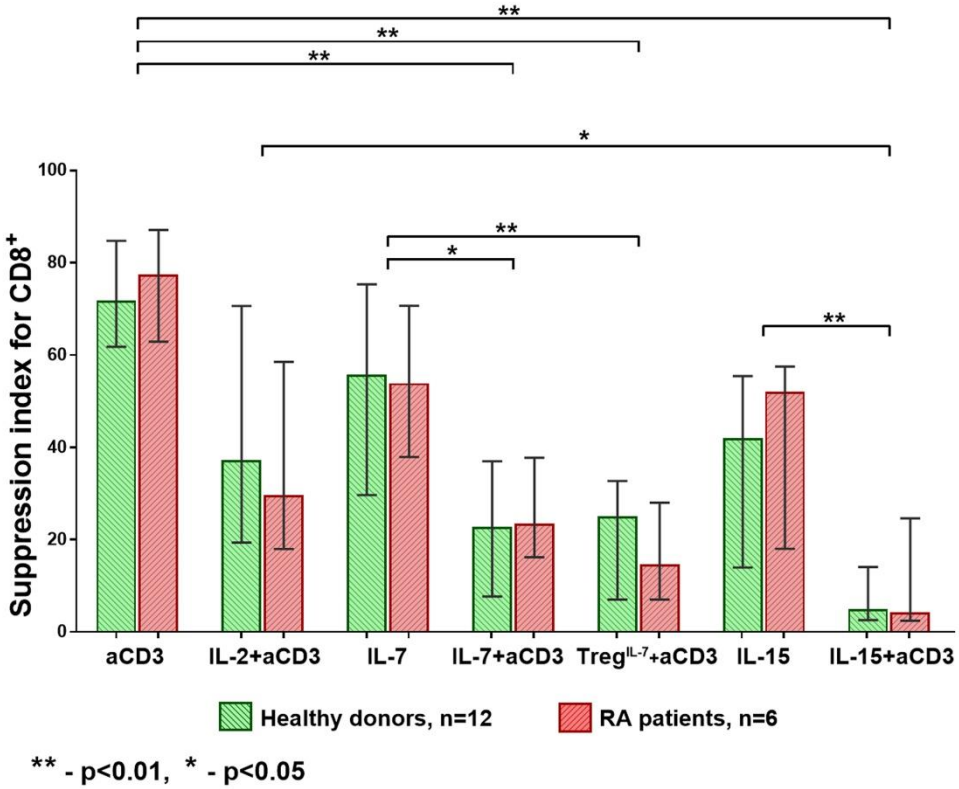


Fig.3. Treg suppression index for CD8$^+$cells. Median, IQR. Comparison of related groups was performed using the Friedman test (ANOVA); post hoc analysis was performed using the Dunn test. Unrelated groups were compared using the Mann-Whitney test; RA – rheumatoid arthritis.

A similar SI pattern was also observed for $CD4^+$ and $CD8^+$ cells in RA patients. The SI in RA patients was significantly reduced when the cells were exposed to IL-7 or IL-15 accompanied by strong TCR stimulation [16] with no significant differences revealed between HD and RA patients in all cultivation conditions (Fig 2, 3). In order to find out whether disease activity or duration have influenced Treg suppressive activity, we additionally enrolled thirteen patients with different illness duration and DAS-28, and assessed the suppressive activity of Treg under anti-CD3+IL-2 stimulation (Fig 4).

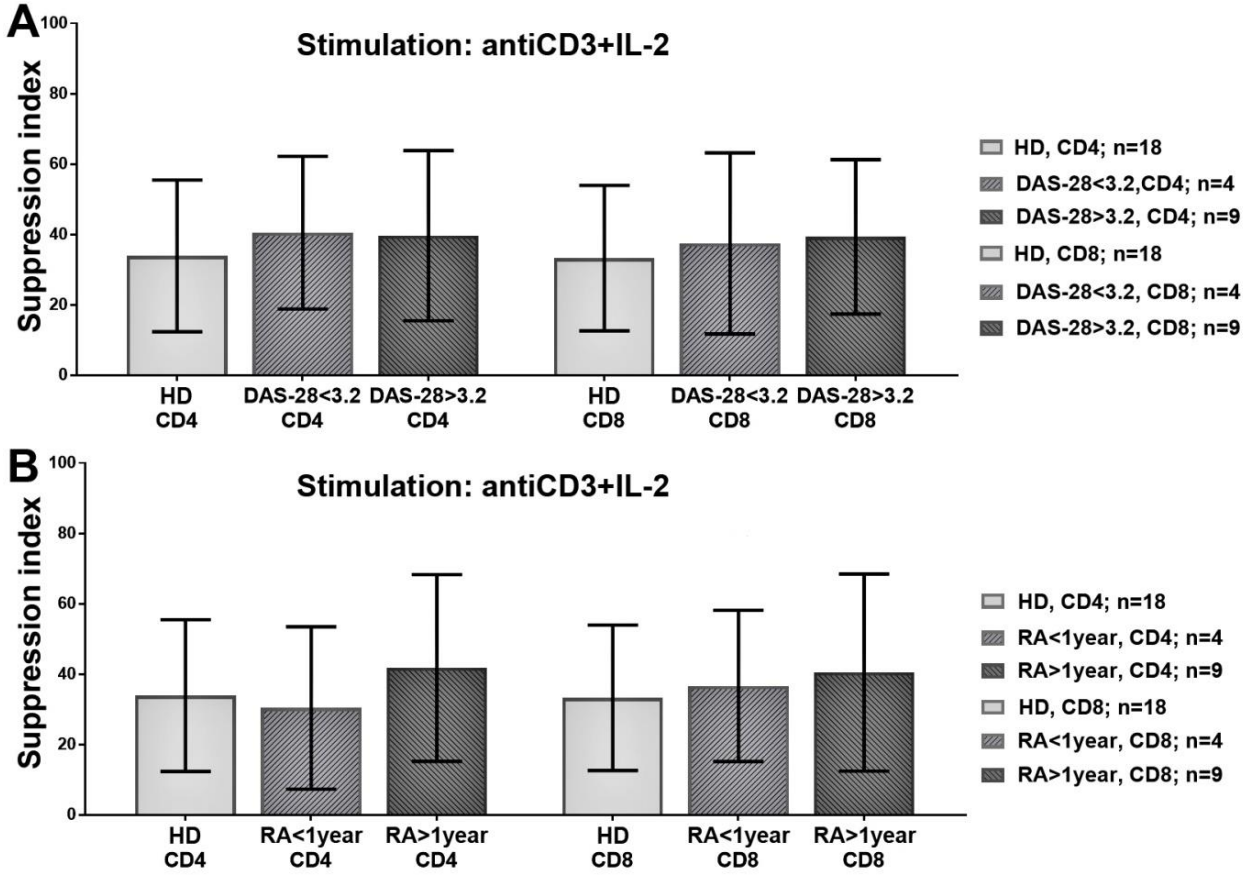


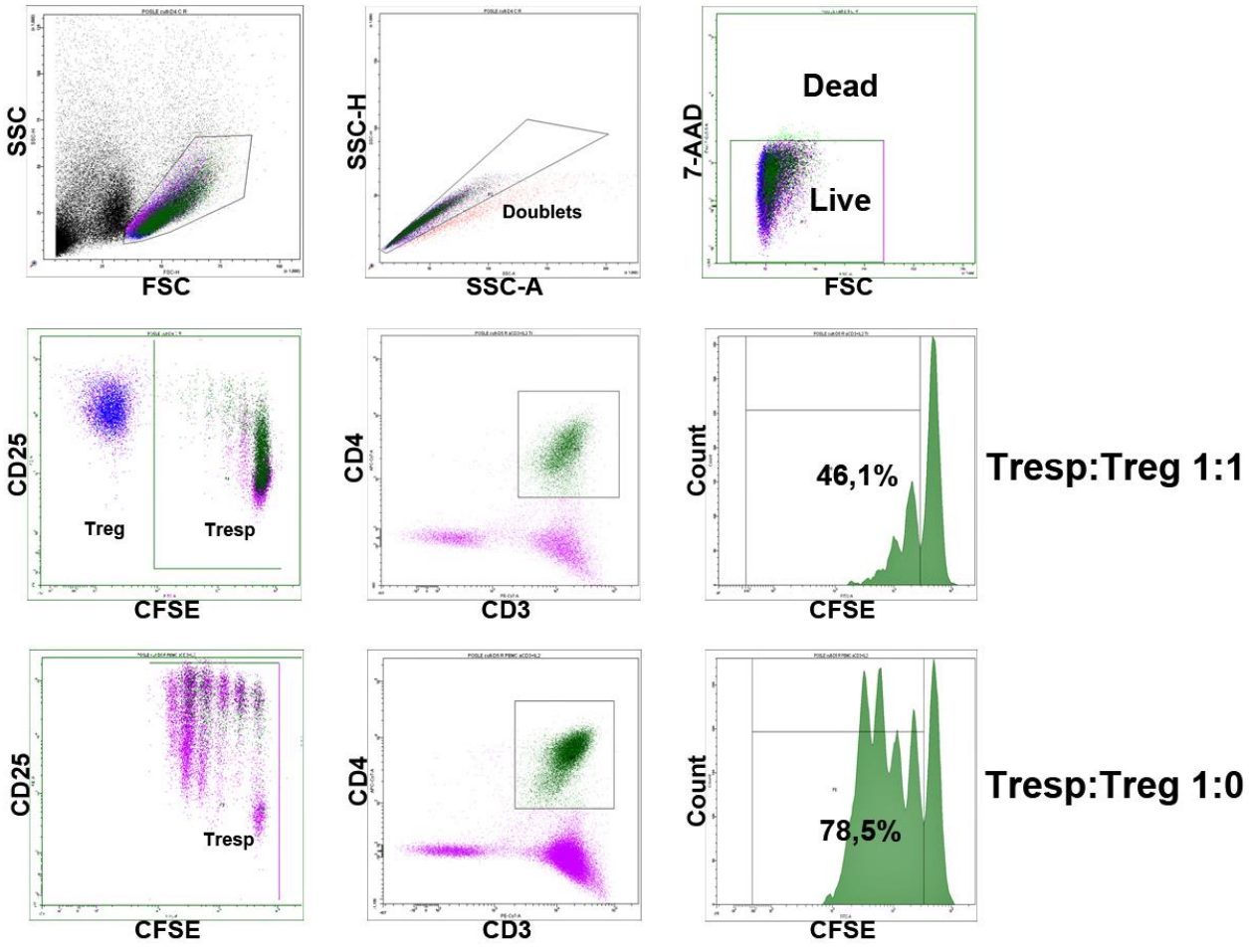
Fig. 4. *Treg suppression index for $CD4^+$ and CD8 cells in HD and RA with different DAS-28 (***A***) and duration of disease (***B***).Mean ± SD. Unrelated groups were compared using the Kruskal-Wallis test; post hoc analysis was performed using the Dunn test; HD – healthy donors, RA – rheumatoid arthritis.*

However, we did not reveal any significant differences in the suppression index of Treg cells between HD and RA patients with different disease duration and DAS-28. It is also worth noting that we did not find any significant correlations between SI and clinical parameters such as ESR or CRP (data not shown). Thus, the total pool of peripheral blood Treg cells in RA patients, independent of disease duration or activity, has same suppression potential as the cells of healthy donors.

The mechanism by which IL-7 or IL-15 in combination with anti-CD3 decrease the suppressive activity of Tregs is unknown. It appears to be the mechanism that directly affects both Tconv and Treg cells which may be confirmed by the experiments when Tregs are pretreated with IL-7. Besides, there is data that strong TCR stimulation may lead to the enhanced Tconv Granzyme-B production accompanied by an increased death of Tregs due to higher contact cytotoxicity [37]. Therefore, not only did we analyse Tregs suppressive activity, but we also evaluated the quantity of dead or damaged cells using 7-AAD as described in the previous study (Fig 5) [38]. However, no

decrease of Tregs quantity under various stimulation conditions was observed (less than 0.5±0.11% of dead Treg cells on average). Thus, the low SI cannot be associated with a decreased Treg number.

It is worth noting that the direct influence of stimulation factors on Tconv could affect the ability of Tregs to inhibit Tconv proliferation. So, the proliferative activity of $CD4^+$ and $CD8^+$ cells significantly varied in different cultivation conditions. As expected, the highest proliferation rate was observed when anti-CD3 was combined with IL-2, IL-7, or IL-15. At the same time, the low proliferation rate was observed when cells were cultivated with IL-7 or IL-15 alone (Fig 6). Such a low proliferation rate is assumed to be an approximation of slow HP, while the high proliferation caused by strong TCR stimulation [16] with HP cytokines (anti-CD3+IL-7 or anti-CD3+IL-15) is likely to imitate the fast HP. It should be noted that no significant differences were found in proliferation of Tconv between donors and RA patients in all cultivation conditions (Fig 6). Despite the high proliferation rate of $CD4^+$ and $CD8^+$ cells stimulated by anti-CD3+IL-2, the SI was also high in both HD and RA. This was not the case for anti-CD3+IL-7 and anti-CD3+IL-15 stimulation indicating that IL-7 and IL-15 are not able to replace IL-2 and cannot effectively support the functional activity of Tregs in conditions close to strong TCR stimulation.



$$\text{Suppression index, SI} = 100 \times \left(1 - \frac{46{,}1}{78{,}5}\right) = 41{,}2$$

Fig.5. Example of the gating strategy for the evaluation of Treg SI for $CD4^+$cells.

Thus, the results obtained indicate that simultaneous action of HP cytokines and strong TCR signal [16] impairs the Tregs capacity to suppress Tconv proliferation.

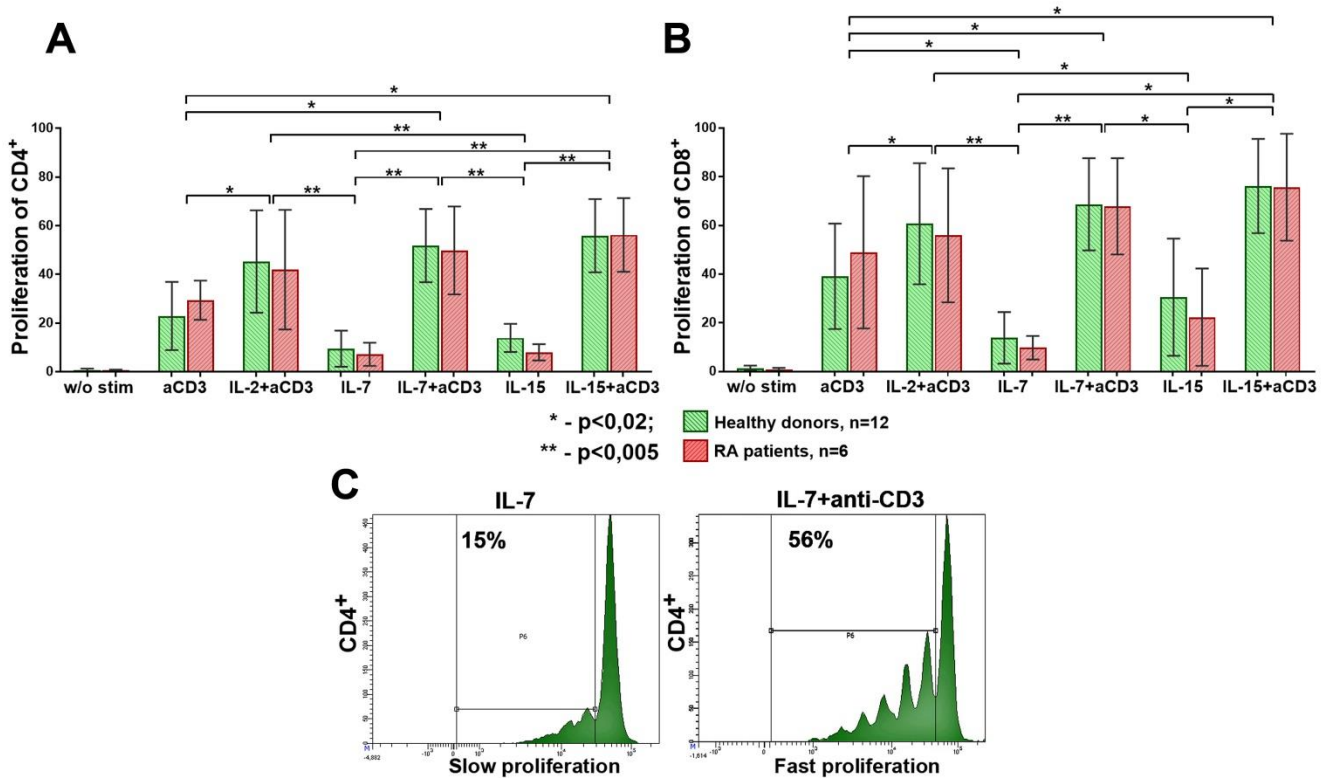


*Fig.6. Proliferation of $CD4^+$ (**A**) and $CD8^+$ (**B**) cells in healthy donors and RA patients,* **C** *– example of slow and fast proliferation (on the example of one of the donors). Mean ± SD. Comparison of related groups was performed using one-way analysis of variance for dependent groups (RM one-way ANOVA); post hoc analysis was performed using Tukey test. Unrelated groups were compared using unpaired Student's t-test; RA – rheumatoid arthritis.*

Treg proliferation caused by HP cytokines is decreased in RA patients

In order to further evaluate the functional activity of Treg cells in RA patients, we again used HP cytokines to examine their proliferation rate. The highest Treg proliferation was observed when the cells were stimulated by anti-CD3+IL-2 with the result being similar in HD and RA groups. However, Tregs proliferation under IL-7 or IL-15 was significantly decreased in the RA group (Fig 7).

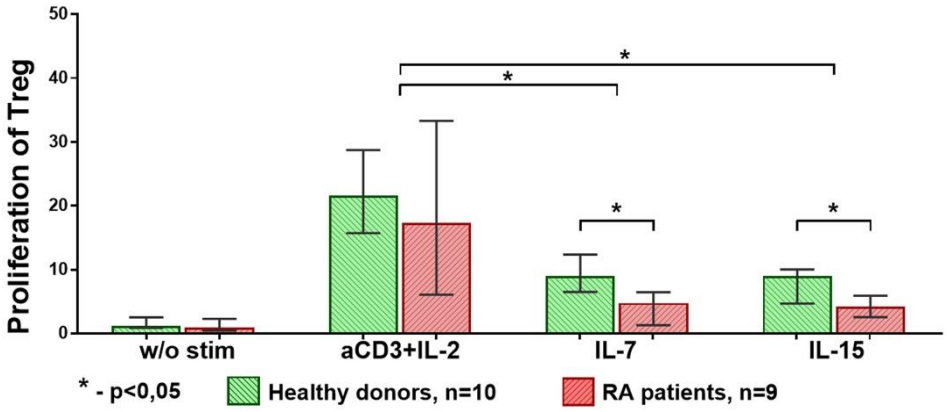


Fig.7. Proliferation of Treg cells in HD and RA. Median, IQR. Unrelated groups were compared using the Mann-Whitney test. Comparison of related groups was performed using the Friedman test (ANOVA); post hoc analysis was performed using the Dunn test; RA – rheumatoid arthritis.

The nature of this phenomenon is unknown and additional research is required. It should note that immunosuppressive therapy could make a significant contribution to decreasing the proliferative activity of Tregs in RA patients; nonetheless, we did not reveal any differences in the proliferation of $CD4^+$ or $CD8^+$ cells between HD and RA patients in all conditions of stimulation.

Mechanisms of Treg suppressive activity

At the next stage of study, we explored the effect of HP cytokines on the Tregs functional molecules expression in vitro. The influence of different conditions of stimulations (w/o stimulations, anti-CD3+IL-2, IL-7, and IL-15) was investigated using purified, CFSE-labeled $CD3^+CD4^+CD25^+Cd127^{lo}$ Treg cells cultivated with autologous PBMC in 1:1 ratio.

First, we assessed TGF-β and IL-10 concentrations in supernatants on the 3rd day (according to the manufacturer's recommendations) (Fig 8). Statistical analysis revealed that anti-CD3+IL-2 stimulation significantly increased TGF-β and IL-10 production in both groups compared to the control levels when no stimulation was applied. Herewith, IL-7 and IL-15 significantly increased TGF-β production in RA group. Moreover, TGF-β and IL-10 production was significantly higher in patients compared to healthy donors under the anti-CD3+IL-2 stimulation.

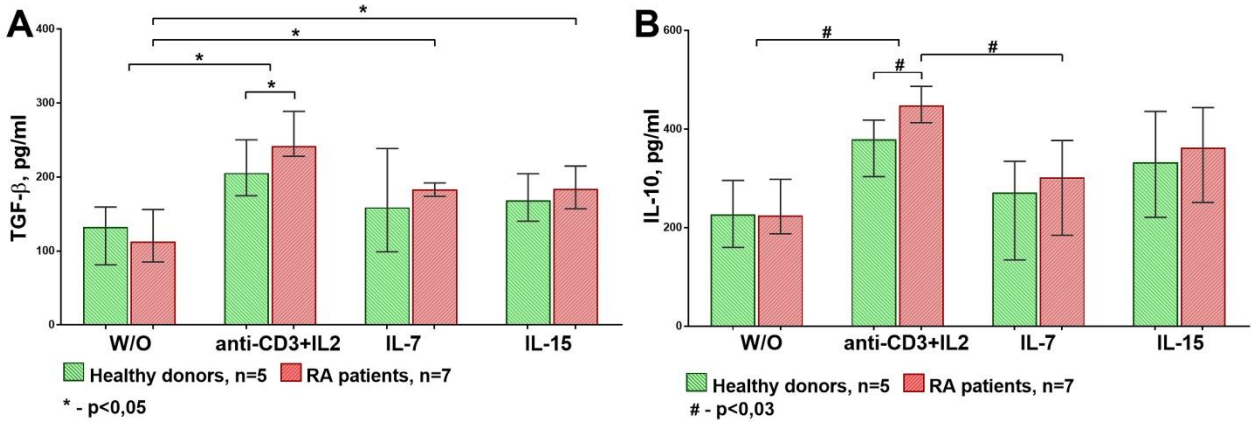


*Fig.8. TGF-β (***A***) and IL-10 (***B***) concentrations in supernatants of Treg: PBMC cultures. Median, IQR. Unrelated groups were compared using the Mann-Whitney test. Comparison of related groups was performed using the Friedman test (ANOVA); post hoc analysis was performed using the Dunn test; RA – rheumatoid arthritis, W/O – without stimulation.*

In addition to the evaluation of TGF-β and IL-10 concentrations in supernatants, we also assessed extracellular expression of PD-L1, CTLA-4, HLA-DR, and CCR4 on Treg cells. The gating strategy is shown in Figure 9. Statistical analysis revealed the following changes (Fig 10):

- Anti-CD3+IL-2 stimulation significantly increased the percentage of $CTLA\text{-}4^+$Treg and MFI of CTLA-4 in both groups.
- Anti-CD3+IL-2 stimulation significantly increased the percentage of $PD\text{-}L1^+$Treg cells in the HD group with and the same tendency (p=0,07, Dunn test) was observed in the RA group. Herewith IL-7 and IL-15 had no impact on percentage of $PD\text{-}L1^+$Treg cells in both groups.
- At the same time all types of stimulation applied (anti-CD3+IL-2, IL-7, IL-15) significantly increased MFI of PD-L1 (Mean Fluorescence Intensity) on Tregs in both groups.
- Anti-CD3+IL-2 and IL-7 significantly increased of percentage of $HLA\text{-}DR^+$Tregs in the HD group, also anti-CD3+IL-2 significantly increased MFI of HLA-DR on Tregs in both groups.

- Anti-CD3+IL-2 significantly increased the percentage of CCR4^{+}Tregs and MFI of CCR4 on Tregs in HD. Herewith, the CCR4^{+}Tregs percentage in RA group was still significantly higher in comparison with HD as in peripheral blood.

Thus, the only difference between HD and RA groups was the CCR4 chemokine receptor expression - is the only receptor for the chemokines CCL22 and CCL17.

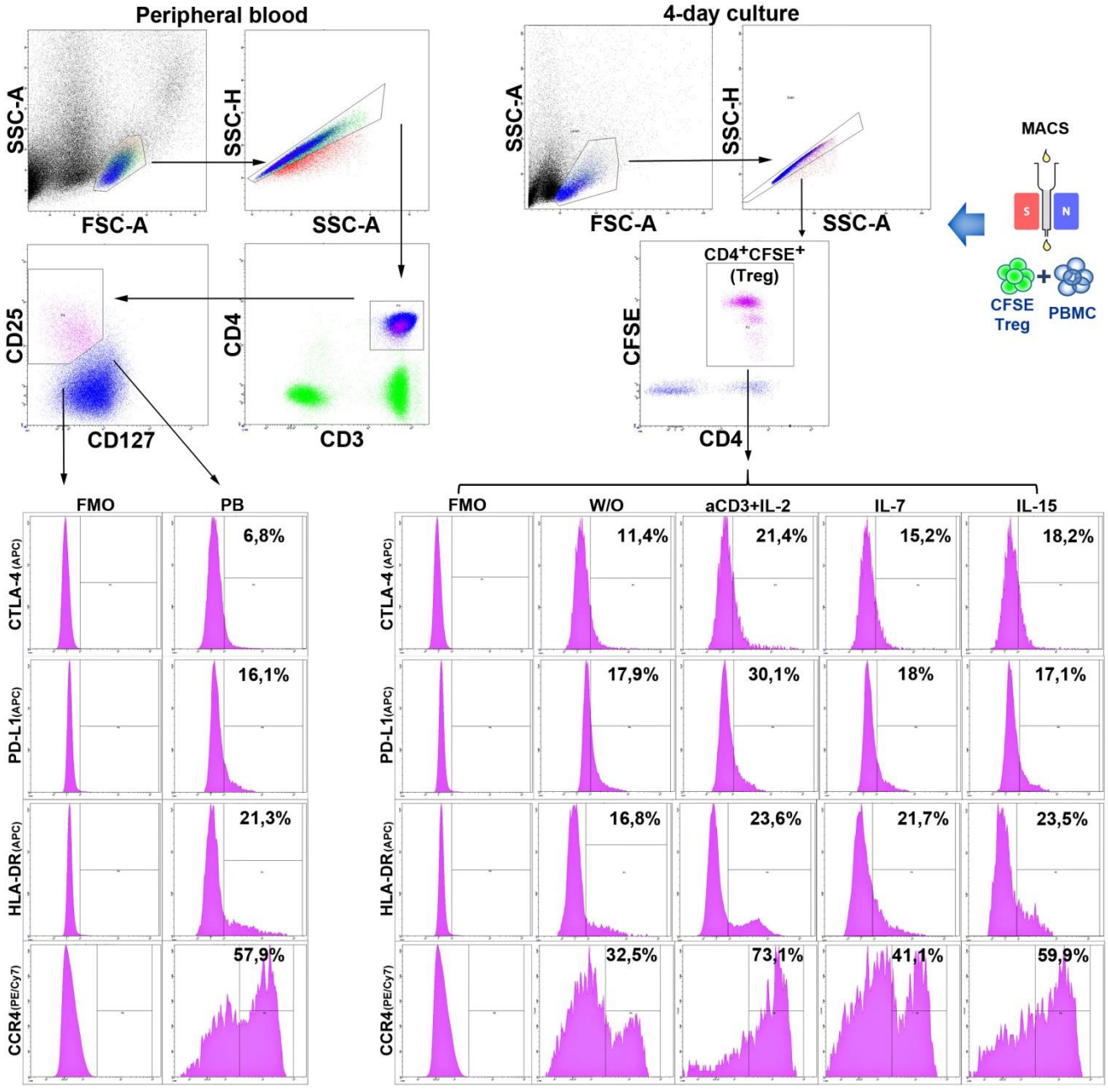


Fig.9. Example of gating strategy for CTLA-4, PD-L1, HLA-DR, and CCR4 expression analysis on the example of one of the RA patients.

Thus, when anti-CD3+IL-2 stimulation was used, Treg cells of RA patients showed a higher ability to produce anti-inflammatory cytokines and were characterized by a greater CCR4 expression than in HD which may indicate their activated status in patients with RA. This data is consistent with the cytometry analysis of Treg cells from peripheral blood conducted before cultivation. It revealed a higher frequency of CCR4^{+}Tregs in peripheral blood accompanied by a lower expression of CTLA-4.

Thus, Treg cells from RA patients demonstrate an effector phenotype and exhibit more potency to produce anti-inflammatory cytokines in vitro under anti-CD3+IL-2.

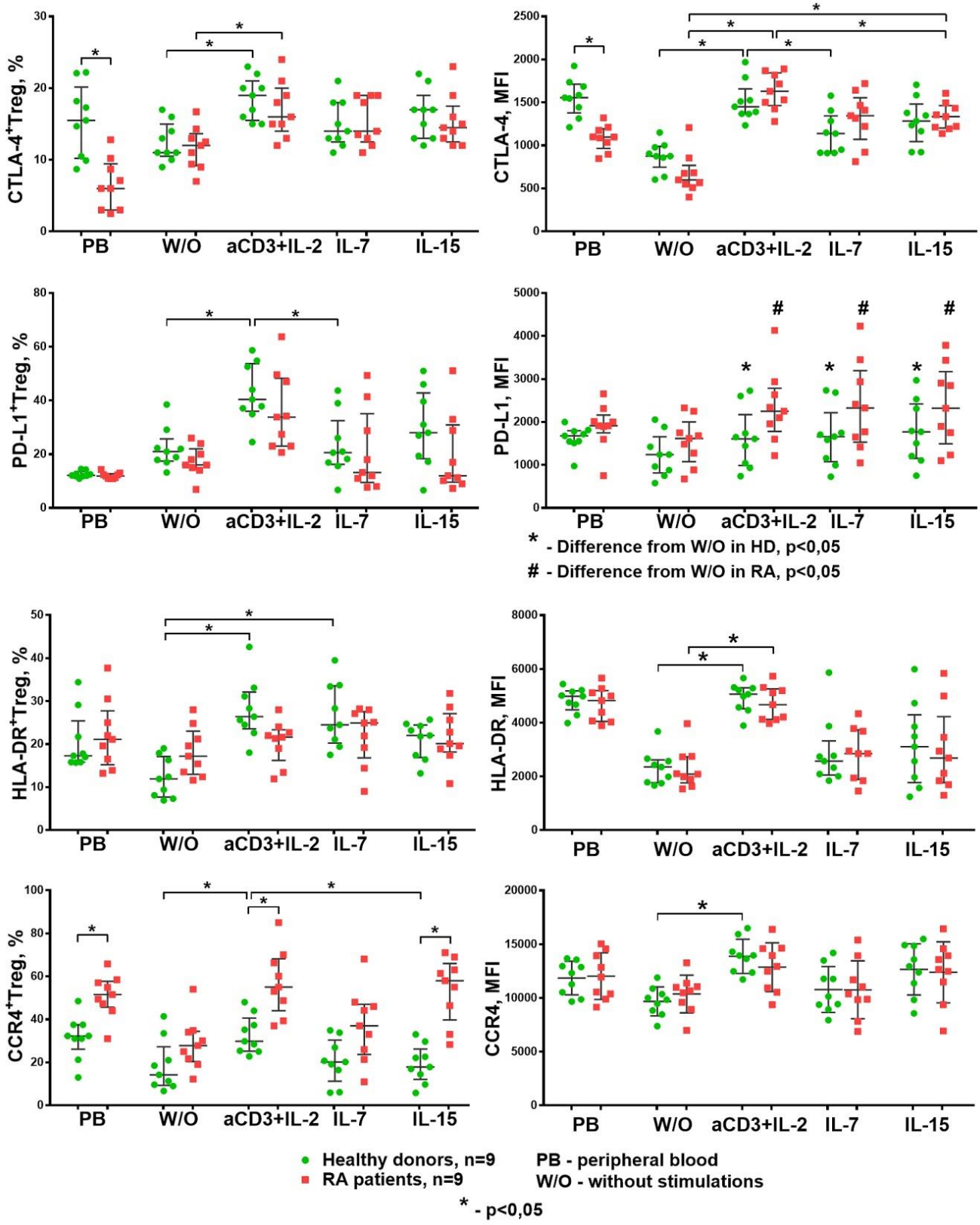


Fig.10. Expression of different functional molecules on Treg cells during cultivation. Median, IQR. Unrelated groups were compared using the Mann-Whitney test. Comparison of related groups was performed using the Friedman test (ANOVA); post hoc analysis was performed using the Dunn test.

Discussion

This study pursued two main objectives: to compare the suppressor activity of Tregs between healthy donors and patients with rheumatoid arthritis and to assess the effect of humoral factors of homeostatic proliferation on the suppressive activity of Treg lymphocytes.

As a result of the experiments, it was not possible to detect any difference in the Tregs suppressive activity between healthy donors and RA patients, which corresponds to the previously published data [30, 32, 39]. This can be regarded as evidence that the suppressive potential of the total Treg pool of patients with RA is preserved at the level of healthy donors with the absence of Treg intrinsic defects. This is confirmed by the studies that revealed no differences between healthy donors and RA patients in terms of gene expression profiles and comprehensive analysis of mi-RNA expression in Treg cells from peripheral blood [32, 40]. Therefore, the resistance of responder cells to suppression may explain impaired Treg suppression of cell proliferation and cytokine production by effector cells from the site of inflammation in RA.

HP cytokines – IL-7 and IL-15, applied separately and, thus, simulating slow proliferation conditions, had no influence on the Treg suppressive activity. However, a combination of IL-7 or IL-15 with anti-CD3 significantly decreased the ability of Tregs to suppress the proliferation of T-responders, both $CD4^+$ and $CD8^+$. As noted above, such stimulation is similar to the conditions of fast homeostatic proliferation in vivo when deep lymphopenia is observed with a large number of free niches simultaneously present. The decrease in the number of producer cells due to lymphopenia results in a relative deficiency of IL-2 which may be an additional factor of Tregs decreased functional activity in vivo. This may lead to the proliferation of various T-cell clones, including self-reactive clones [18, 19, 41]. As noted earlier, this is due to the competition between different T-lymphocytes clones for proliferation signals with the signal transmitted via TCR playing here the most important role. And in this competition, the advantage belongs to those lymphocyte clones whose TCR have a higher affinity to bind the peptide in the MHC complex. Given a limited number of foreign antigens presented in the body simultaneously and a continuous cross-presentation process, the majority of the presented antigens are epitopes of self-proteins. That is why it can be assumed that the majority of cells that enter the homeostatic proliferation are Tconv with a higher TCR affinity for self-peptides along with a small number of clones specific for foreign antigens that are currently present in the body. Thus, as a result of rapid homeostatic proliferation self-reactive clones of T-cells are selected to proliferate. This leads to a decrease in TCR diversity and increases the risk of autoimmunity. Although the difference between the slow and fast HP is to some extent arbitrary, the fast HP may lead to qualitative changes in the immune system. If the slow HP may be considered as an analog of physiological regeneration, then the fast HP may be identified with the emergency tissue regeneration damaging its structure and functions. Thus, we revealed the inability of Tregs to suppress fast proliferation of Tconv cells caused by strong TCR signal and HP cytokines, which may represent an additional mechanism of link between homeostatic proliferation and autoimmunity.

The ability of Tregs to suppress the proliferation of Tconv in response to strong stimulation of TCR [16] was also reduced by the Treg pretreatment with IL-7 indicating its direct negative influence on the Tregs suppressive function. This is consistent with the previously obtained data [42, 43], which showed a decrease in the expression of *EOMES* and *NF-κB* genes and an increase in the expression of *IL-6* and *INF-γ* genes in Treg cells under the influence of IL-7. However, there is still not enough

reliable evidence to connect the change in the expression of these genes with a decrease in the Treg suppressive activity, which sets the ground for future research.

To study in more detail the effect of HP cytokines on Treg suppressor activity, we evaluated the expression of several functional molecules on the Treg surface. It is well known that one of the important Treg suppressor molecules is CTLA-4. It binds to the CD80/86 co-stimulation molecules on dendritic cells and removes pMHC-II complexes from the dendritic cells surface through trans-endocytosis [44]. Another molecule mediating suppressor functions of Tregs is PD-L1; it interacts with PD-1 on the activated effector cells and causes its anergy and apoptosis or even induces Tregs de novo [45]. The chemokine receptor CCR4 is the only receptor for the chemokines CCL22 and CCL17. Both ligands are known to evoke chemotaxis of CCR4-bearing Treg cells to draining lymph nodes and to mature dendritic cells expressing CCL17 and CCL22 that this is critical for Treg cell suppressive function in vivo [46, 47]. Besides, $CCR4^+$Treg cells are characterized by activated phenotype and high suppressive activity [48]. HLA-DR is one of the key cell surface molecules expressed on antigen-presenting cells, but HLA-DR expression is also expressed on activated Treg cells with high FoxP3 expression [30]. Such Tregs with HLA-DR expression exhibit early contact suppression activity [49]. So, the analysis of the HP humoral factors influence on the expression of the functional molecules by Treg lymphocytes showed that the greatest suppressor potential Tregs acquire under the influence of the combination of anti-CD3+IL-2. It significantly increased the percentage of Tregs expressing PD-L1, CTLA-4, HLA-DR, and CCR4, as well as the membrane expression density (MFI) of PD-L1 and CTLA-4 molecules, while IL-7 and IL-15 increased only the expression density (MFI) of the PD-L1. The observed increase in the percentage of $PD\text{-}L1^+$ and $CTLA\text{-}4^+$ Tregs and the expression density of the PD-L1 and CTLA-4 molecules (MFI) may indicate an increase in the suppressor potential of Tregs under the anti-CD3+IL-2 stimulation. The revealed pattern also corresponds to the published data on the effect of cytokines with a common γ-chain on the PD-L1 expression by $CD4^+$ lymphocytes [50]. However, given that IL-7 and IL-15 could not increase the number of $PD\text{-}L1^+$ and $CTLA\text{-}4^+$ Tregs, it may be concluded that HP cytokines cannot provide the same level of Treg suppressive activity as anti-CD3+IL-2. Given that the CCR4 molecule can also be used as a potential marker of effector Treg lymphocytes [48], the increased expression of this molecule, together with the CTLA-4 and HLA-DR, indicates the ability of anti-CD3+IL-2 stimulation to cause Tregs activation and transition into effector Treg cells.

Importantly, there was no difference in the expression of all analyzed molecules between the groups of donors and patients, except for the CCR4 – with its percentage being significantly higher in the peripheral blood of RA patients and when anti-CD3+IL-2 and IL-15 stimulation was applied. Similar results were obtained for the IL-10 and TGF-β production. Anti-CD3+IL-2 stimulation significantly increased their concentration in supernatants of both HD and RA groups. In addition, concentrations of these cytokines were significantly higher in the RA group in comparison with the HD in this type of stimulation. Thus, the RA group showed an increased percentage of $CCR4^+$Tregs and a higher production of IL-10 and TGF-β, which may indicate the compensatory activated state of Treg cells in rheumatoid arthritis that could be caused by physiological mechanisms or the medications applied for the treatment. Therefore, the persistence of inflammation in synovia in rheumatoid arthritis is likely to be caused by the resistance of T-responders to Treg suppressive signals [35, 39, 51].

Conclusion

The study of the effect of HP humoral factors on the Treg suppressor activity showed that Tregs can effectively suppress the proliferation of T-responders caused by IL-7 and IL-15. However, when these HP cytokines were accompanied by strong TCR stimulation with anti-CD3, the ability of Tregs to suppress the proliferation of Tconv lymphocytes was significantly reduced, possibly playing one of the key roles in case of deep lymphopenia followed by the fast HP. Moreover, the observed decrease in Treg suppressor activity can occur in respect to a narrow spectrum of self-reactive clones of Tconv lymphocytes that are able to receive a strong signal from TCR even in case of slow HP in mild lymphopenia. All that can lead to a decrease in the diversity of TCR repertoire during homeostatic proliferation regardless of its intensity. Consequently, it may increase the risk of autoimmune disease development. Thus, we have identified a new potential mechanism for the development of autoimmune diseases, which consists of a decrease in the suppressor activity of T-regulatory cells to self-reactive T-lymphocytes under conditions of homeostatic proliferation.

In addition, we have shown that HP cytokines IL-7 and IL-15 cannot provide the same level of Treg suppressive activity as IL-2. Therefore, the decrease in the number of producer cells due to lymphopenia results in a relative deficiency of IL-2, and it is an additional factor of Tregs decreased functional activity in vivo during lymphopenia.

We have revealed the preserved suppressive potential of the general pool of Treg cells in patients with rheumatoid arthritis. However, this does not exclude the presence of any other defects at the clonal level. We suppose that in the future, the identification of specific T-lymphocytes clones, which are involved in the violation of self-tolerance, will make it possible to use individual approaches in the therapy of patients with RA, targeting these clones with monoclonal antibodies or CAR-T-lymphocytes.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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