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ВЛИЯНИЕ ГОМЕОСТАТИЧЕСКИХ ЦИТОКИНОВ – IL-7 И IL-15 НА Т-РЕГУЛЯТОРНЫЕ КЛЕТКИ *IN VITRO*

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Резюме. Цитокины IL-7 и IL-15 являются наиболее важными гуморальными факторами, обеспечивающими восстановление пула Т-лимфоцитов после лимфопении в процессе гомеостатической пролиферации (ГП). Однако неизвестно, могут ли эти цитокины обеспечивать гомеостатическое поддержание и пролиферацию Т-регуляторных клеток (Treg). Также, учитывая связь между гомеостатической пролиферацией и развитием аутоиммунных заболеваний, интересно, могут ли цитокины ГП вызывать переход Treg-клеток в Th17-лимфоциты. Поэтому целью данного исследования было изучить влияние гуморальных факторов ГП (IL-7 и IL-15) на Treg-клетки *in vitro*. В исследовании использовалась периферическая кровь 22 здоровых доноров. Фракцию МНК получали центрифугированием в градиенте плотности фиколла. Для стимуляции пролиферации использовались цитокины ГП – IL-7 и IL-15, а также комбинация IL-2 с анти-CD3-антителами. Интенсивность пролиферации Treg оценивалась с помощью проточной цитометрии с использованием CFSE в общих культурах МНК по фенотипу $CD3^+CD4^+CD25^+FoxP3^+$, а также в чистой популяции Treg-клеток $CD3^+CD4^+CD25^+CD127^{lo}$, полученной с помощью иммуномагнитной сепарации с использованием набора MACS Treg Isolation Kit. Также во время культивирования оценивалась экспрессия основного транскрипционного фактора Th17-лимфоцитов – RORγt в $CD3^+CD4^+CD25^+FoxP3^+$-лимфоцитах. Было обнаружено, что IL-7 и IL-15 могут поддерживать Treg-клетки численно и по фенотипу, поддерживая экспрессию FoxP3 в Treg-клетках. При этом, в отличие от цитокинов ГП, стимуляция IL-2 + анти-CD3 индуцировала экспрессию FoxP3 *de novo* в $CD4^+$ лимфоцитах. Также было обнаружено, что IL-7 и IL-15 могут вызывать пролиферацию Treg-клеток, однако она значительно менее интенсивна по сравнению со стимуляцией IL-2 + анти-CD3. При этом цитокины ГП не обладали способностью индуцировать экспрессию RORγt как в Treg клетках, так и в $CD4^+$ лимфоцитах. Таким образом, было показано, что IL-7 и IL-15 потенциально могут участвовать в поддержании общего пула клеток Treg во время лимфопении, когда возникает дефицит IL-2, и не индуцируют экспрессию RORγt. Однако, как эти цитокины влияют на функциональную активность Treg-клеток, остается неясным и требует дальнейших исследований.

Ключевые слова: Т-регуляторные клетки, IL-7, IL-15, гомеостатическая пролиферация, RORγt, FoxP3

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INFLUENCE OF HOMEOSTATIC CYTOKINES – IL-7 AND IL-15 ON T-REGULATORY CELLS *IN VITRO*

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Abstract. Cytokines IL-7 and IL-15 are the most important humoral factors providing T-conventional cell pool reconstitution during homeostatic proliferation caused by lymphopenia. However, whether these cytokines can provide homeostatic maintenance and proliferation of T-regulatory (Treg) cells is largely unknown. Considering the association between homeostatic proliferation and the development of autoimmunity, we decided to investigate the ability of these factors to cause differentiation of Treg-cells into Th17-lymphocytes. Therefore, the purpose of this study was to investigate the influence of humoral factors of homeostatic proliferation (IL-7 and IL-15) on Treg-cells *in vitro*. The study used peripheral blood sampled from 22 healthy donors. PBMC fraction was isolated by Ficoll density gradient centrifugation. Proliferation was induced by IL-7, IL-15, and by a combination of IL-2 with anti-CD3-antibodies. The proliferation intensity of Tregs was evaluated by flow cytometry using CFSE in PBMC cultures by phenotype $CD3^+CD4^+CD25^+FoxP3^+$ and in the previously purified population of $CD3^+CD4^+CD25^+CD127^{lo}$-cells. In this case Treg-cells were obtained by immunomagnetic separation from PBMCs using a MACS Treg Isolation Kit. Also, the RORyt expression in $CD3^+CD4^+CD25^+FoxP3^+$-cells was evaluated during cultivation. Here, we have shown that IL-7 and IL-15 could support Treg-cells by number and phenotype. Also, we revealed that these factors provide FoxP3 expression in Treg-cells; meanwhile, stimulation with IL-2 + anti-CD3 can also cause induction of FoxP3 expression *de novo* in conventional $CD4^+$ cells. Also, we have shown that IL-7 and IL-15 can cause lower-intensity proliferation of Treg-cells in comparison with IL-2 + anti-CD3. Herewith homeostatic cytokines didn't have the ability to induce RORyt expression in both T-regulatory cells and $CD4^+$ conventional T-lymphocytes. Thus, it has been shown that IL-7 and IL-15 can potentially participate in maintaining the total pool of Treg-cells during lymphopenia, when IL-2 deficiency occurs, without causing the induction of RORyt expression. However, how homeostatic cytokines affect the functional activity of Treg-cells remains unclear and requires further investigation.

Keywords: T-regulatory cells, IL-7, IL-15, homeostatic proliferation, RORγt, and FoxP3

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Introduction

T-regulatory (Treg) cells are well known to play an important role maintaining autotolerance to self-antigens. Treg-cells have high inhibition potential due to the wide spectrum of suppression such as contact and distant mechanisms. The main factors providing Treg homeostasis are IL-2, "tonic" TCR signaling (TCR – T-cell receptor), and costimulation signals [10]. The importance of these factors for maintenance of the Treg population was shown in different animal models and in humans [7, 12, 15]. In the last 20 years, the disturbance in IL-2/IL-2R signaling or lack of costimulation cues was demonstrated to decrease Treg-cell functional activity and cause diverse autoimmune disorders [11]. It is worth noting that the main transcriptional factor of Treg-cells is FoxP3, which accounts for Treg-cell suppression activity and expression of multiple functional molecules driven by relevant genes [6]. Thus IL-2, TCR signaling, or costimulation signals take a part in providing the expression of FoxP3 in Treg-cells. Taking into account that $CD4^+$- and $CD8^+$-lymphocytes are the main producers of IL-2 for Treg-cells, it can be assumed that deficiency of this cytokine during lymphopenia due to the lack of cell producers can negatively impact on the homeostasis of the Treg pool [11]. Indeed, several studies demonstrate the negative influence of lymphopenia on the Treg population associated with the lack of FoxP3 expression and decreased suppression activity of ex-FoxP3-cells [3, 9]. The lack of FoxP3 expression in Treg is known to be associated with differentiation to Th17-lymphocytes due to the induced RORγt expression [2]. The reconstitution of the T-cell pool by adoptive transfer prevents the negative influence of lymphopenia by increasing IL-2 production. The important role of IL-7 and IL-15 in T-cell repopulation during lymphopenia is well known [1, 5]; however, how these cytokines affect the homeostasis of the Treg-cell population and whether they have a potential to support FoxP3 expression and result in Treg proliferation remains understudied.

Thus, the goal of this study was to evaluate the influence of IL-7 and IL-15 on FoxP3 and RORγt expression and assess their potential to cause Treg-cell proliferation.

Materials and methods

This study was performed by using peripheral blood samples collected from 22 apparently healthy donors aged from 27 to 70 years (49±13 years). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. Phenotyping of Treg was performed by flow cytometry. All monoclonal antibodies were purchased from BioLegend (San Diego, CA, USA): CD3 by PE/Cy7, CD4 by APC/Cy7, CD25 by APC, and FoxP3 by PE were used for $CD3^+CD4^+CD25^+FoxP3^+$ phenotype; $CD3^+CD4^+CD25^+CD127^{lo}$ phenotype: CD3 by PE/Cy7, CD4 by APC/Cy7, CD25 by PE, and CD127 by PerCP/Cy5.5. Intracellular staining was performed by using True-Nuclear Transcription Factor Buffer Set according to the manufacturer's instructions (BioLegend). For evaluation of RORγt expression, monoclonal antibodies were used (RORγt by PerCP; R&D, Minneapolis, MN, USA). Isotype and FMO controls were used for the estimation of RORγt expression. Treg-cells were pre-stained by CFSE for the proliferation assay. The CFSE-stained PBMCs were cultured in 48-well flat-bottom plates (TPP, Trasadingen, Switzerland) for 4 days by placing 1.5 million cells per ml, and then $CD3^+CD4^+CD25^+FoxP3^+$-cell proliferation was evaluated by flow cytometry. The CFSE-stained Treg-cells were also cultured with PBMC in 200 μl of total volume in a 1-to-1 ratio (30,000 Treg per 30,000 PBMC) for 4 days in round-bottomed plates. In this case, Treg-cells were obtained by immunomagnetic separation from PBMCs using a MACS Treg Isolation Kit (Miltenyi Biotec, Auburn, CA, USA). The population was isolated to contain $CD3^+CD4^+CD25^+CD127^{lo}$-phenotype (purity was 93±4%). The cell culture was performed in RPMI-1640 media supplemented with 10% FCS and antibiotics. Three types of stimulation protocols were used: IL-2 (100 U/ml; NPK BIOTECH, coupled with anti-CD3 antibodies (1 μg/ml; Sorbent, Pasadena, TX, USA), IL-7 (50 U/ml; MyBiosource, San Diego, CA, USA), and IL-15 (50 U/ml; MyBiosource). The cells were cultured without stimulation as a negative control.

Statistical analysis was performed by GraphPad Prism software (v. 7.03; GraphPad, La Jolla, CA, USA). Nonparametric methods were applied for descriptive statistics (median with interquartile range). For multiple independent groups, the comparison was conducted with the Kruskal–Wallis test, while for multiple dependent groups comparison was performed by using the Friedman test. Post hoc analysis was performed by using Dunn's test. The differences were considered significant at $p < 0.05$.

Results and discussion

After cell culture, the percentage of $CD25^+FoxP3^+$-lymphocytes was higher at all stimulation conditions than in the control stimulation-free setting (3.5±1.7%), whereas the percentage of Treg-cells was higher by adding IL-2 + anti-CD3 (9.7±3.4%) *vs* IL-7 (7.9±2.8%) or IL-15 (7.7±3.1%). At the same time, the amount of Treg-cells was lower without stimulation than in the samples from peripheral blood before cultivation (6.2±1.5%). This result agrees with our previous data [8]. To directly evaluate the influence of homeostatic cytokines – IL-7 and IL-15 on the expression of FoxP3, we assessed the FoxP3 MFI in Treg-cells at different stimulation conditions (Figure 1). We revealed a significantly decreased FoxP3 expression in cells without stimulation. Meanwhile, stimulation with homeostatic cytokines – IL-7 and IL-15 supported the expression of FoxP3 in Treg-cells. It should note that the maximal FoxP3 expression was upon IL-2 + anti-CD3 stimulation, exceeding the values for peripheral blood.

The significant decrease in the percentage of $CD25^+FoxP3^+$-cells and FoxP3 MFI expression in the control cells without stimulation was due to decreased CD25 (IL-2R) expression in the absence of the key stimulation factors [4]. Here, stimulation with IL-7 or IL-15 could similarly support the Treg amount and FoxP3 expression albeit to a lesser extent than IL-2 + anti-CD3, which suggests that the cytokines IL-7 and

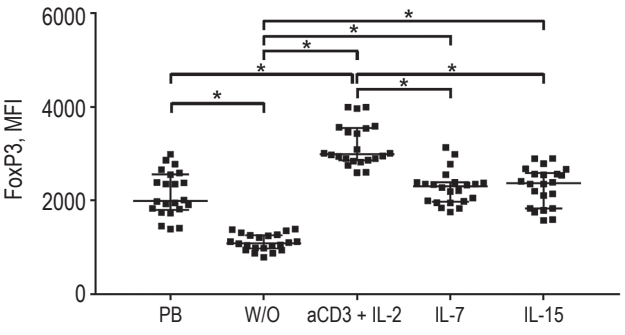

Figure 1. MFI of FoxP3 in Treg-cells in various cell culture conditions. Expression of FoxP3 in Treg-cells significantly decrease during culture without stimulation. Meanwhile, stimulation with IL-7 or IL-15 supports FoxP3 expression in Treg cells even to a lesser extent than IL-2 + anti-CD3

Note. n = 22, data are presented as the median and interquartile range of 25-75%, the comparison was performed by using the Friedman test, post hoc analysis – Dunn's test. MFI, Mean Fluorescent Intensity; PB, peripheral blood; W/O, without stimulation; aCD3, anti-CD3 antibodies. *, $p < 0.001$

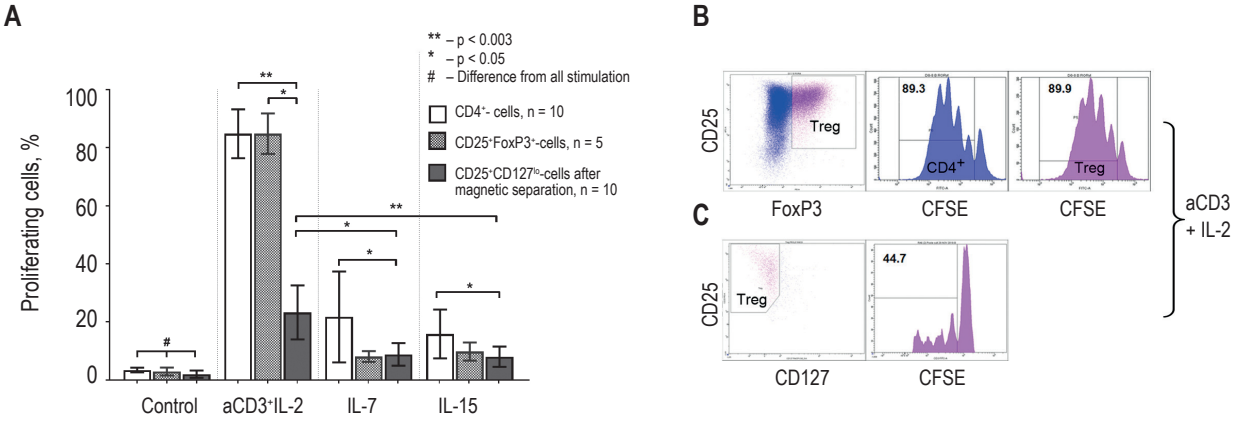

Figure 2. A, the level of proliferation of CD4+ and Treg-cells under various culture conditions. IL-2 + anti-CD3 stimulation, in contrast to effects of IL-7 or IL-15, can induce FoxP3 expression in conventional CD4+ T-cells. B, a gating strategy for CD25+FoxP3+-cells in total PBMC cultures and proliferation of CD4+ and CD25+FoxP3+-cells in PBMC cultures. C, a representative proliferation of Treg-cells obtained by magnetic separation

Note. The data are presented as a median and interquartile range of 25-75%. The comparisonwas performed by using the Kruskal–Wallis test for unrelated groups and the Friedman test for related groups. The post hoc analysis in both cases used Dunn's test.

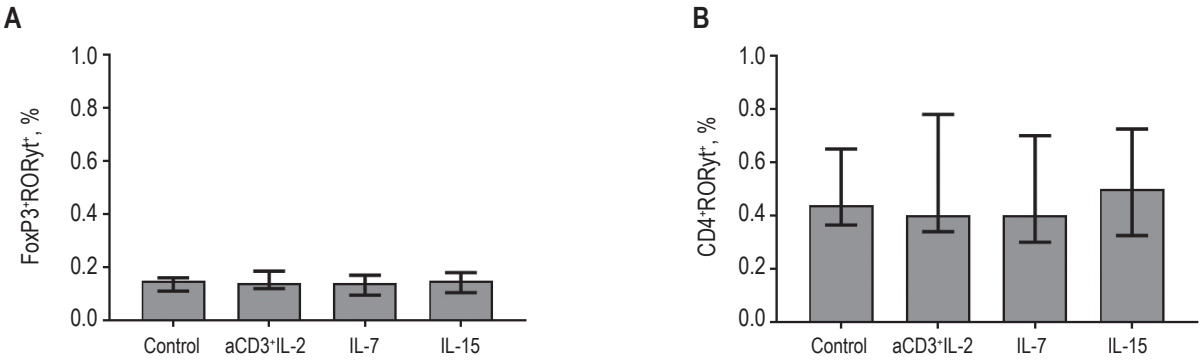

Figure 3. Percentage of FoxP3+RORγt+-lymphocytes gated on CD25+FoxP3+-cells (A) and the percentage of CD4+RORγt+-lymphocytes gated on CD4+-cells (B) in PBMC cultures; n = 5. Homeostatic cytokines – IL-7 and IL-15 caused no induction of RORγt in Treg and conventional CD4+-lymphocytes *in vitro*

Note. The data are presented as a median and interquartile range of 25-75%. The comparison used the Kruskal–Wallis test.

IL-15 can potentially support the expression of FoxP3 in Treg-cells *in vivo* during lymphopenia.

The induction of Treg-lymphocytes from effector CD4+ cells is well known to occur under certain conditions. Such induced Treg-lymphocytes are not mainly "true" regulatory T-lymphocytes because they are prone to lose FoxP3 expression and may differentiate into pathogenic Th-17-lymphocytes in some situations [14]. Thus, it seems important to determine an underlying mechanism resulting in increased percentage of CD25+FoxP3+-lymphocytes in PBMC cultures occurred exposed to these stimulation conditions: i.e., either due to proliferation or induction. Hence, we compared the proliferation of CD25+FoxP3+-lymphocytes in PBMC cultures with the proliferation of previously purified Treg-cells. Figure 2 shows the data obtained for the proliferation of $CD3^+CD4^+CD25^-FoxP3^-$ effector cells, $CD3^+CD4^+CD25^+FoxP3^+$ Treg-cells in PBMC cultures and for "pure" $CD3^+CD4^+CD25^+CD127^{lo}$Treg-lymphocytes.

The differences revealed in the proliferation of Treg-lymphocytes in PBMC cultures from the proliferation of Treg-cells isolated by magnetic separation are associated with the induction of *de novo* CD25+FoxP3+-lymphocytes from conventional CD4+T-cells under stimulation by IL-2 combined with anti-CD3. However, this effect was not observed during the stimulation with IL-7 and IL-15. Figure 2 shows that the proliferation of Treg-cells was significantly

lower than that of $CD4^+$ lymphocytes under all stimulation conditions. Nonetheless, cytokines IL-7 and IL-15 could induce the proliferation of Treg-cells, although to a lesser extent, than the stimulation with IL-2 + anti-CD3, which may be important under lymphopenia.

In the next step of our study, we tested whether homeostatic cytokines can induce Treg differentiation into Th-17-lymphocytes. The analysis of RORγt expression revealed no increased percentage of transient $FoxP3^+ROR\gamma t^+$ and $CD4^+ROR\gamma t^+$-lymphocytes after any culture conditions (Figure 3), which suggests that the cytokines IL-7 and IL-15 cannot induce *in vitro* expression of the transcription factor RORγt, at least in healthy donors.

A significantly higher level of Treg-cell proliferation under the influence of the IL-2 + anti-CD3 combination *vs* IL-7 and IL-15 was detected, which was expected due to being associated with the nature of such stimuli. According to some assumptions, stimulation with anti-CD3 antibodies is an analog of strong TCR stimulation, while IL-2 is the pivotal cytokine involved in activating and maintening Treg pool by accounting for STAT5 expression [14]. Thus, the data obtained allow us to conclude that the homeostatic cytokines – IL-7 and IL-15 can effectively support Treg-lymphocyte quantity and FoxP3 expression at least *in vitro*. These results are generally consistent with the most published data, which indicate about potential of common γ-chain-driven cytokines to positively influence Treg-cell homeostasis particularly due to activation of the STAT5 signaling pathway [13]. No increase in RORγt induction was detected under IL-7 and IL-15 stimulation, which confirms the ability of IL-7 and IL-15 to maintain the homeostasis of the Treg population resulting in no induction of Th-17-cells.

Conclusion

In general, these results indicate the ability of cytokines IL-7 and IL-15 to provide homeostatic maintenance of the Treg population under IL-2 deficiency. However, the lower proliferation of Treg-lymphocytes compared to $CD4^+$-lymphocytes may lead to a delayed recovery of Treg pool and may impair immune balance under lymphopenia. Herewith, it is worth noting that humoral factors IL-7 and IL-15 do not increase RORyt expression or contribute to Treg differentiation towards $exFoxP3^-RORyt^+$-cells.

Authors' contributions

Daniil Shevyrev performed all *in vitro* experiments, analysed the data, and wrote the manuscript. Vladimir Kozlov conceived and designed experiments, gained financial support, and finally approved the manuscript.

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