ЗНАЧЕНИЕ NOTCH-СИГНАЛИНГА В РЕГУЛЯЦИИ ДИФФЕРЕНЦИРОВКИ Treg-ЛИМФОЦИТОВ У БОЛЬНЫХ С ИНФИЛЬТРАТИВНЫМ ТУБЕРКУЛЕЗОМ ЛЕГКИХ

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Резюме. В современной литературе активно накапливаются данные о роли регуляторных Т-лимфоцитов (Treg) в иммунопатогенезе туберкулеза. Подавляющее действие Treg-клеток на пролиферацию, функциональную активность Th1-лимфоцитов и антигенпрезентирующих клеток позволяет рассматривать данную популяцию в качестве возможной мишени модуляции иммунного ответа у больных туберкулезом. Сигнальный путь Notch принимает участие в регуляции экспрессии транскрипционного фактора FoxP3 и, следовательно, способен поддерживать супрессорную активность Treg-лимфоцитов. Ключевая роль в функционировании сигнального каскада Notch принадлежит ферменту γ-секретазе, отщепляющему внутриклеточный домен рецептора (Notch ICD) с последующим образованием комплекса, регулирующего дифференцировку клеток. Активно изучаемым ингибитором γ-секретазы является DAPT – N-[N-(3,5-дифторфенацетил)-L-аланил]-S-фенилглицин трет-бутиловый эфир). Материалом для исследования служили мононуклеарные лейкоциты, выделенные из крови больных лекарственно-чувствительным и лекарственно-устойчивым туберкулезом легких методом градиентного центрифугирования до начала противотуберкулезной терапии. Клетки культивировали в условиях стимуляции антигенами микобактерий туберкулеза CFP10-ESAT6 или с добавлением в инкубационную среду ингибитора γ-секретазы (DAPT) в дозах 5 мкМ/л и 10 мкМ/л в комбинации с CFP10-ESAT6 при 37 °С и 5% CO_2 в течение 72 ч. Количество Treg-лимфоцитов оценивали методом проточной цитофлуориметрии путем определения экспрессии поверхностного рецептора CD4 (FITC) и внутриклеточного транскрипционного фактора FoxP3 (PE). В интактных культурах клеток больных туберкулезом легких относительное количество Treg-лимфоцитов статистически значимо ($p < 0,001$) превышало аналогичные показатели у здоровых доноров. Стимуляция клеток антигенами CFP10-ESAT6 сопровождалась увеличением доли $CD4^+FoxP3^+$-клеток в обеих группах больных туберкулезом. Добавление в инкубационную среду ингибитора γ-секретазы в концентрации 5 мкМ/л не приводило к статистически значимым изменениям количества Treg-лимфоцитов. Увеличение концентрации DAPT до 10 мкМ/л сопровождалось уменьшением количества Treg-лимфоцитов

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по сравнению с соответствующими показателями при стимуляции антигенами CFP10-ESAT6 во всех группах обследуемых. Вне зависимости от условий культивирования число $CD4^+FoxP3^+$-клеток у пациентов с лекарственной устойчивостью микобактерий превышало их количество у больных лекарственно-чувствительным туберкулезом легких. Угнетение сигнального пути Notch при помощи ингибитора γ-секретазы (DAPT) в концентрации 10 мкМ/л способствует снижению количества Treg-лимфоцитов у больных лекарственно-чувствительным и лекарственно-устойчивым туберкулезом легких. Уменьшение числа Treg-лимфоцитов при помощи ингибитора γ-секретазы подтверждает значение сигнального каскада Notch как потенциально возможной мишени для коррекции иммунносупрессорной активности Treg-лимфоцитов и патогенетической терапии туберкулеза.

Ключевые слова: Notch, Т-регуляторные клетки, дифференцировка, лекарственная устойчивость, туберкулез легких, гамма-секретаза, DAPT

SIGNIFICANCE OF NOTCH SIGNALING IN THE REGULATION OF Treg LYMPHOCYTE DIFFERENTIATION IN PATIENTS WITH INFILTRATIVE PULMONARY TUBERCULOSIS

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Abstract. Data on the role of regulatory T lymphocytes (Treg) in the immunopathogenesis of tuberculosis are actively accumulating in the current literature. The overwhelming effect of Treg cells on the proliferation, functional activity of Th1 lymphocytes and antigen-presenting cells allows to consider this population as a possible target of modulation of the immune response in patients with tuberculosis. The Notch signaling pathway participates in the regulation of FoxP3 transcription factor expression and, therefore, is capable of supporting suppressor activity of Treg lymphocytes. A key role in the functioning of the Notch signaling cascade belongs to the enzyme γ-secretase that cleaves the intracellular domain of the receptor (Notch ICD), with the subsequent formation of a complex that regulates cell differentiation. The actively studied inhibitor of γ-secretase is DAPT — N-[N-(3.5-difluorophenacetyl)-L-alanyl]-S-phenylglycine tert-butyl ester). Mononuclear leukocytes isolated from the blood of patients with drug-sensitive and drug-resistant pulmonary tuberculosis by gradient centrifugation before the start of anti-tuberculosis therapy were used as the material for the study. The cells were cultured under conditions of stimulation with *Mycobacterium tuberculosis* antigens CFP10-ESAT6 or with the addition of γ-secretase inhibitor (DAPT) at doses of 5 μM/L and 10 μM/L in combination with CFP10-ESAT6 at 37 °C and 5% CO_2 for 72 h to the incubation medium. The number of Treg lymphocytes was assessed by flow cytofluorimetry by determining the expression of the CD4 surface receptor (FITC) and the intracellular transcription factor FoxP3 (PE). In intact cell cultures of pulmonary tuberculosis patients, the relative number of Treg lymphocytes was statistically significantly ($p < 0.001$) higher than that of healthy donors. Stimulation of cells with CFP10-ESAT6 antigens was accompanied by an increase in the proportion of $CD4^+FoxP3^+$ cells in both groups of tuberculosis patients. Addition of γ-secretase inhibitor at a concentration of 5 μM/L to the incubation medium did not lead to statistically significant changes in the number of Treg lymphocytes. The increase in DAPT concentration up to 10 μM/L was accompanied by a decrease in the number of Treg lymphocytes in comparison with the corresponding indices upon stimulation with CFP10-ESAT6 antigens in all groups of the subjects. Regardless of cultivation conditions, the number of $CD4^+FoxP3^+$ cells in patients with drug-resistant mycobacteria exceeded their number in patients with drug-sensitive pulmonary tuberculosis. Inhibition of the Notch signaling pathway by a γ-secretase inhibitor (DAPT) at a concentration of 10 μM/L contributed to a decrease in the number of Treg lymphocytes in patients with drug-sensitive and drug-resistant pulmonary tuberculosis. Reduction of Treg lymphocyte number by γ-secretase inhibitor confirms the importance of Notch signaling cascade as a potential target for correction of Treg lymphocytes immunosuppressive activity and pathogenetic therapy of tuberculosis.

Keywords: Notch, T regulatory cells, differentiation, drug resistance, pulmonary tuberculosis, gamma-secretase, DAPT

Introduction

Protective control over the infectious process caused by *Mycobacterium tuberculosis* is ensured by the cooperative interaction of multiple immunocompetent cells, realized through juxtacrine and paracrine mechanisms [12]. Data on the role of regulatory T lymphocytes (Treg) in pathogenesis of immune response at pulmonary tuberculosis (PT) are actively accumulating in the modern literature [2, 3]. The immunosuppressive function of Treg cells is implemented through the secretion of cytokines (IL-10, TGF-β and IL-35), suppression of expression of costimulation molecules (CD80 and CD86) necessary for activation of helper T cells type 1 (Th1), and induction of granzyme-dependent apoptosis of target cells by dendritic cells [2]. Increased number of Treg lymphocytes in patients with PT correlates with multidrug resistance of the pathogen, bacillary load, and is also accompanied by chronicization and aggravation of the pathological process [8]. The overwhelming effect of Treg-cells on the proliferation and functional activity of Th1 lymphocytes allows us to consider this population as a possible target of modulation of the immune response in patients with tuberculosis [5]. The Notch signaling pathway has been shown to be an important mechanism of intercellular signaling that regulates innate and adaptive immune response responses [7, 10].

The literature presents data indicating the importance of the Notch molecular cascade in the pathogenesis of tuberculosis. It has been established that stimulation of mouse macrophages is accompanied by an increase in the expression of Notch receptors and its ligands, Jagged 1, Dll 1 and Dll 4. The progressive course of tuberculosis infection is associated with an increase in the number of monocytes/macrophages and dendritic cells expressing Notch-receptor ligand – Dll 4 [1, 9, 11]. The results of experiments on cell lines demonstrate the critical importance of Notch-1 signaling pathway in the regulation of transcription factor FoxP3 expression and, as a consequence, the maintenance of immunosuppressive function of Treg cells [1].

A key role in the functioning of the Notch signaling cascade belongs to γ-secretase, an enzyme that cleaves the intracellular domain of the receptor (Notch ICD), with the subsequent formation of a complex that regulates cell differentiation. A known inhibitor of γ-secretase, which is being actively studied, is DAPT – N-[N-(3.5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester [4, 6, 10, 13].

Materials and methods

The study involved 15 patients with newly diagnosed infiltrative pulmonary tuberculosis. The middle age of the patients was 45.4±6.58 years. Depending on the sensitivity of mycobacteria to anti- tuberculosis drugs, all patients were divided into two groups: 8 patients with drug-sensitive PT and 7 patients excreting mycobacteria resistant to at least isoniazid and rifampicin. The control group consisted of 8 healthy volunteers of comparable sex and age. The material for the study was whole peripheral venous blood taken before the start of anti-tuberculosis therapy. Mononuclear leukocytes were isolated from blood by gradient centrifugation (ρ = 1.077 g/mL). *Mycobacterium tuberculosis* antigens CFP10-ESAT6 (Diakintest, Generium, Russia) were added to the incubation medium at a dose of 10 μg/mL or γ-secretase inhibitor (DAPT, Tocris Bioscience, UK) at doses of 5 μM/L and 10 μM/L in combination with CFP10-ESAT6. Cells were cultured in complete RPMI-1640 medium with L-glutamine (BioloT LLC, Russia) at 37 °C and 5% CO_2 for 72 h. The number of Treg lymphocytes was estimated by flow cytofluorimetry by determining the expression of CD4 surface receptor (FITC, BD Biosciences, USA) and intracellular transcription factor FoxP3 (PE, BD Biosciences, USA). The results were processed using a statistical software package IBM SPSS Statistics 20. The Shapiro-Wilk test was used to check the correspondence of the data to the normal distribution law. Significance of differences in quantitative data was assessed using Mann–Whitney U nonparametric test. Wilcoxon test was used to assess the significance of differences in dependent data within the group. The results of statistical analysis were considered significant at the $p < 0.05$ level.

Results and discussion

The key role in the coordinated antigen-specific immune response to *Mycobacterium tuberculosis* belongs to homeostasis and dynamic interaction between dendritic cells, macrophages and various populations of T lymphocytes: Th1, Th2, Th17, Treg. The question about the molecular mechanisms of immunoregulatory imbalance remains open. One of the factors of pathological process progression in tuberculosis is excessive immunosuppressive activity of regulatory T cells. By inhibition of antigen-dependent differentiation and activation of apoptosis of Th1 lymphocytes, as well as suppressive influence on antigen-presenting cells, Treg population contributes to suppression of immune response and induces more severe course of disease with long-term persistence of pathogen.

The study showed that in patients with infiltrative drug-sensitive (DS) and drug-resistant (DR) PT in intact cultures the relative number of Treg lymphocytes ($CD4^+FoxP3^+$) was statistically

TABLE 1. RELATIVE CONTENT OF Treg LYMPHOCYTES IN PERIPHERAL BLOOD (% OF TOTAL LYMPHOCYTES) IN PATIENTS WITH INFILTRATIVE PULMONARY TUBERCULOSIS, Me ($Q_{0.25}$-$Q_{0.75}$)

Cultivation conditions in vitro	Healthy donors	Patients with infiltrative pulmonary tuberculosis	
		Drug-sensitive	Drug-resistant
Treg lymphocytes ($CD4^+FoxP3^+$)			
Intact culture	2.58 (2.37-3.15)	5.31 (5.24-5.39) $p_1 < 0.001$	4.88 (4.63-5.11) $p_1 < 0.001$ $p_4 = 0.022$
With added antigens (CFP10-ESAT6)	2.63 (2.43-3.21) $p_2 = 0.007$	5.7 (5.65-5.76) $p_1 < 0.001$ $p_2 = 0.012$	5.09 (4.78-5.22) $p_1 < 0.001$ $p_2 = 0.012$ $p_4 = 0.001$
With added antigens and DAPT (5 μM/L)	2.61 (2.41-3.2)	5.69 (5.62-5.73) $p_1 < 0.001$	5.09 (4.76-5.20) $p_1 < 0.001$ $p_4 = 0.001$
With added antigens and DAPT (10 μM/L)	2.39 (2.27-3.02) $p_3 = 0.008$	4.91 (4.86-5.00) $p_1 < 0.001$ $p_3 = 0.012$	4.15 (4.06-4.30) $p_1 < 0.001$ $p_3 = 0.012$ $p_4 = 0.001$

Note. p_1, level of statistical significance of differences compared with similar parameters in healthy donors; p_2, compared with baseline parameters (in intact cell culture); p_3, compared with parameters during antigen stimulation; p_4, compared with parameters in patients with drug-sensitive pulmonary tuberculosis.

significantly ($p < 0.001$) higher than that in healthy volunteers (Table 1). We may assume that increased number of $CD4^+FoxP3^+$ cells causes suppression of Th1-mediated immune response and may promote pathological process progression or, on the contrary, prevent formation of hyperergic immune reaction and lung tissue damage.

After stimulation of cell cultures with CFP10-ESAT6 antigens, we registered an increase in the proportion of $CD4^+FoxP3^+$ cells relative to initial values in both groups of patients and healthy donors (Table 1).

The addition of a γ-secretase inhibitor (DAPT) at a concentration of 5 μM/L to the cell suspension in combination with CFP10-ESAT6 antigens was not accompanied by statistically significant changes in the number of Treg lymphocytes both in PT patients and healthy donors (Table 1).

Increasing the DAPT dose up to 10 μM/L resulted in a decrease in the number of $CD4^+FoxP3^+$ cells compared to the corresponding indices upon stimulation with CFP10-ESAT6 antigens in all groups of subjects (Table 1).

It should be noted that regardless of cultivation conditions the number of Treg lymphocytes in patients with drug-resistant mycobacteria exceeded ($p_4 = 0.001$) their number in DS PT patients (Table 1). The obtained data may indicate a more pronounced inhibition of the functional activity of $CD4^+$Th1 lymphocytes, which provide cellular effector responses, in patients with DR PT.

Conclusion

Inhibition of the Notch signaling pathway by a γ-secretase inhibitor (DAPT) at a concentration of 10 μM/L contributes to a decrease in the number of Treg lymphocytes in patients with DS and DR PT. The reduction of Treg lymphocytes number by γ-secretase inhibitor confirms the importance of Notch signaling cascade as a potential target for correction of Treg lymphocytes immunosuppressive activity and pathogenetic therapy of tuberculosis.

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