Rapamycin reduced inhibition of Vγ1 γδ T cells towards Vγ4 γδ T cells through down-regulating IL-4

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Abstract

To explore the role of mTOR signaling in Vγ1 γδ T cells, and the effect of mTOR signal changes of Vγ1 γδ T cells in antitumor effects of Vγ4 γδ T cells, purified Vγ1 γδ T cells were cultured with 10 nM rapamycin, and were detected its proliferation, apoptosis and key cytokine expression level. Rapamycin-treated Vγ1 γδ T cells were co-cultured with Vγ4 γδ T cells to characterize the effect of Vγ1 γδ T cells to Vγ4 γδ T cells. The results showed that mTOR signaling of Vγ1 γδ T cells was restrained after rapamycin treatment, with no change in proliferation and apoptosis. However, the GATA-3 and IL-4 expression of Vγ1 γδ T cells were restrained, led to reduced inhibition towards antitumor effect of Vγ4 γδ T cells. In conclusion, Rapamycin reduced IL-4-mediated Vγ4 γδ T cell tumor inhibition though down-regulating IL-4 expression of Vγ1 γδ T cells.

Keywords

Rapamycin, Vγ1 γδ T cells, anti-tumor effect.

1. Introduction

γδ T cell has unique biological function that can be directly activated by antigens instead of being presented antigens by MHC molecules, and could produce a lot of cytokines in short term. γδ T cells have multiple subsets which distributes in different tissues, Vγ1 and Vγ4 γδ T cells are two main subsets distributed in spleen and lymph nodes in mice. γδ T cells play an important role in many diseases. In children with primary nephrotic syndrome, IL-17 + γδ T cells and 23R+γδ T cells are involved in the pathogenesis through affecting Th17 / Treg cells balance[1]. CD5 NK1.1+γδ T cells participated in early protection in listeria infection via Bcl11b-indepent pathway[2]. Hong MJ[3] found that mice infected with influenza and exposed to chronic smoke would recover slowly, besides, IL-17 expressiong was upregulated in lung, thus inhibiting γδ T cell-mediated antiviral responses. Gao YF[4-7] found that such as γδ T cells played an important role of protection in tumor disease through early infiltration and providing early sources of IFN - γ. In additon, some studied show that tumor-infiltrating γ δ T cells showed good prognosis phenomenon[8]. Our previous study found that Vγ1 and Vγ4 γδ T cells play a different role in the tumor, Vγ4 γδ T cells recognize tumor cells by NKG2D and TCR , and express perforin and IFN-γ to kill tumor cells[9]. However, Vγ1 γδ T cells did not show an anti-tumor effect, and can suppressed the anti-tumor function of Vγ4 γδ T cells via expressing IL-4[10].

mTOR is a key protein that participates in many physiological and pathological processes[11], which is mainly in the form of mTORC1 and mTORC2, and is sensitive to the inhibitor Rapamycin[12]. In tumor-related studies, mTOR is active in tumor cells and has regulation effects in tumor proliferation[13, 14]. In the study of T cells, mTOR mediated the differentiation and function of CD4+T cells, CD8+T cells, Treg cells and Th17 cells by regulating related metabolism[15-18]. However, there's less research about mTOR signals in γδ T cells, especially the regulatory effect of mTOR in the antitumor function of γδ T cells is unclear. In our previous study, when Vγ4 γδ T cells treated with rapamycin, the mTOR signaling in Vγ4 γδ T cells is restrained, with downregulating

phosphorylation levels of downstream ribosomal protein S6 and upregulating phosphorylation levels of STAT5, led to the up-expression of NKG2D and TNF-a, eventually the anti-tumor effects enhance[19]. However, the function of mTOR signaling in Vγ1 γδ T cells is unknown. According to previous research, we suggest mTOR signaling in Vγ1 γδ T cells also have relevant regulatory function.Therefore, the purpose of this paper is to explore the function of mTOR signaling in Vγ1 γδ T cells, and its effect in the antitumor function of Vγ4 γδ T cells.

2. Materials and Methods

2.1 Mice

Female C57BL/6J wildtype mice were purchased from the experimental animal center of the academy of military medical sciences, and raised in the laboratory animal center of college of life sciences of Nankai university.

2.2 γδ T Cells Culture and Purification

Splenic cells of mice were cultured with plate-coated anti-Vγ1/Vγ4 mAb(10 g/ml), anti-CD28 mAb(1 g/ml) and rmIL-2(2ng/ml) for 5d for expansion of Vγ1/Vγ4 γδ T cells, respectively. Expanded Vγ1/Vγ4 γδ T cells were enriched by strepavedin conjugated magnet beads and biotin-conjugated anti- Vγ1/ Vγ4 antibodys, respectively. FACS was used to detect the purification of enriched cells.

2.3 Rapamycin Treatment and Co-culture of Vγ1 and Vγ4 γδ T cells

Rapamycin was disolved in DMSO with an concentration of 1 for following experiments. Enriched Vγ1/ Vγ4 γδ T cells were cultured with rapamycin(10nM) in the presence of IL-2(2ng/ml) for 24h, then γδ T cells were collected and washed by medium or PBS for 3 times to remove residual rapamycin and then used for following experiments. Rapamycin-treated Vγ1 γδ T cells were co-cultured with purified Vγ4 γδ T cells (1.25×105 cells : 1.25×105 cells in a total volume of 500 l) in the presence of IL-2(2ng/ml) for 24h, then γδ T cells were collected for subsequent experiments.

2.4 Co-culture of γδ T Cells and B16 tumor cells

Aforesaid prepared γδ T cells(5×104cells) were cultured with B16 tumor cells(1×104cells), which are at logarithmic phase, in a total volume of 200 l in 96-well plate， IL-2(2ng/ml) was also needed. After 24h, tumor cells was calculated using trypan blue.

2.5 Flow cytometric analysis

γδ T cells were collected and adjusted in a concentration of 5×106 cells/ml, added with PMA(50ng/ml), Ionomycin(1 g/ml) and Golgi-Plug(1:1000) for 6h activation culture. After that, cells were stained with fluorescently labeled anti-Vγ1/ Vγ4 Abs. 2% formaldehyde and 0.5% saponin were then mixed with cells for fixation and permeabilization step by step, subsequently, cells were stained with fluorescently labeled cytokine-specific antibodys. Foxp3 Staning Buffer Set (eBioscience) was used for transfactor staining and Phosflow reagents(BD Biosciences) was used for S6 protein phosphorylation staining, as instructed. Stained cells was detected by flow cytometry.

2.6 Statistical Analysis

Data was analyzed by GraphPrism 5 software and represented as means ± SD. A two-tailed Student's t-test was used to analyze the differance between groups. Statistical significance: *P<0.05, **P<0.01, ***P<0.001 and ns means no significance.

3. Results

3.1 Rapamycin Treatment Reduce pS6 of Vγ1 γδ T cells

Purified Vγ1 γδ T cells were continued to be cultivated for 24 h, and then collected to detect the purificaion by FACS. As shown in Fig.1 A, the purification of Vγ1 γδ Tcells is up to 96% and is

suitable for following experiments. Purified Vγ1 γδ T cells(3.5×105 cells/well) were then cultured with 10nM rapamycin and vihecle(DMSO), cells number was counted after 24h. Vγ1 γδ T cells proliferated to nearly 5×105 cells and no significant differances between two groups, this result indicates that rapamycin do not suppress the proliferation function of Vγ1 γδ T cells(Fig.1 B), besides, do not induce the apoptosis of Vγ1 γδ T cells(Fig.1 C). Supsuqently, we detected the phosphorylation of S6, a ribosome protein of downstream mTOR signaling. Compared with vehicle group, the pS6 of rapamycin-treated Vγ1 γδ T cells is downregulated, this sugguest that mTOR signaling was suppressed(Fig.1 D), consisting with previous studies.

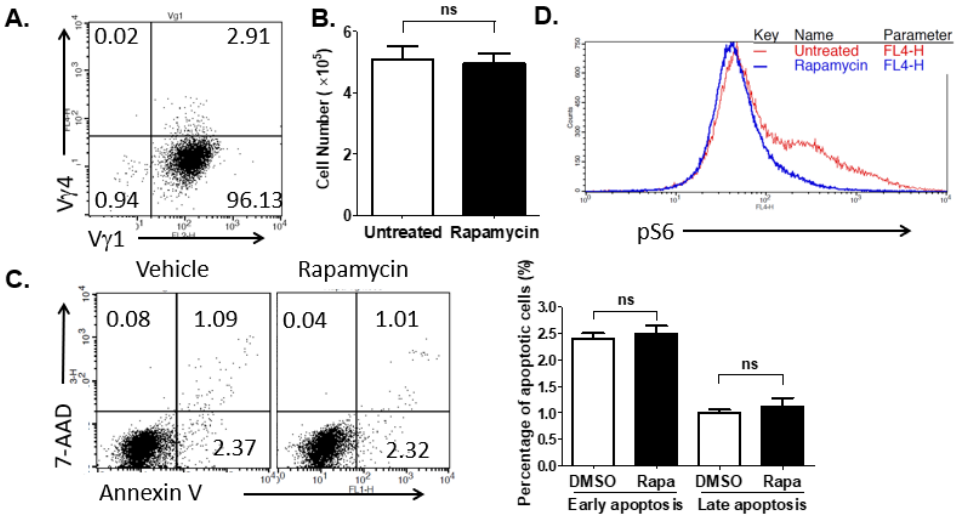

Fig. 1 Rapamycin inhibits mTOR signaling of Vγ1 γδ T cells

A. The purification of Vγ1 γδ T cells is good; B. the proliferation of Vγ1 γδ T cells shows no change; C. rapamycin do not induce apoptosis of Vγ1 γδ T cells; D. rapamycin reduce S6 phosphorylation of Vγ1 γδ T cells.

3.2 Rapamycin Treatment Reduce the suppressing function of Vγ1 γδ T cells

Rapamycin or vehicle-treated Vγ1 γδ T cells and Vγ4 γδ T cells were co-cultured with B16 tumor cells, and tumor cells were counted after 24h. Results shows that both Vγ1 and Vγ4 γδ T cells have anti-tumor effects, and rapamycin-treated Vγ4 γδ T cells seems to have more powerful anti-tumor function, consisiting with our previous research. However, rapamycin treatment do not affect the anti-tumor function of Vγ1 γδ T cells(Fig.2 A). To explore the effect of rapamycin-treated Vγ1 γδ T cells on Vγ4 γδ T cells, we mixed two kinds of cells for 24h culture, and supsequently added B16 tumor cells to the culture complex for 24h, then tumor cells were counted. We found that Vγ1 γδ T cells could significantly inhibits the anti-tumor effect of Vγ4 γδ T cells, but the suppression would be reduced after rapamycin treatment, resulted in recovering anti-tumor effect of Vγ4 γδ T cells(Fig.2 B). These results indicated that rapamycin treatment alleviate the suppressing function of Vγ1 γδ T cells towards Vγ4 γδ T cells.

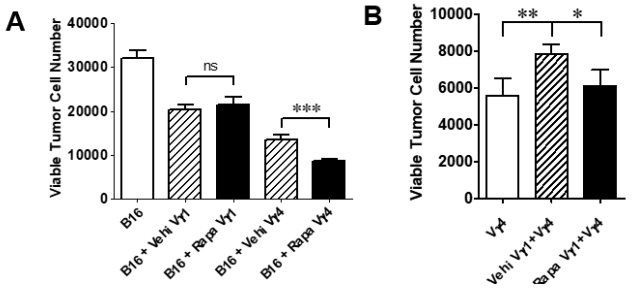

Fig. 2 Rapamycin treatment alleviate the inhibition of Vγ1 γδ T cells towards Vγ4 γδ T cells

A. the anti-tumor effect of Vγ1 γδ T cells shows no changge; B. Rapamycin-treated Vγ1 γδ T cells alleviate the suppression towards Vγ4 γδ T cells.

3.3 Rapamycin Treatment Suppress GATA-3 and IL-4 Expression of Vγ1 γδ T cells

We further explore the mechanism in the process of reduced suppression of rapamycin-treated Vγ1 γδ T cells. According to our previous research, Vγ1 γδ T cells suppressed Vγ4 γδ T cells through expressing IL-4. Thus, we detected the IL-4 levels of supernatant of cell culture complex. The IL-4 levels of rapamycin-treated Vγ4 γδ T cells slightly reduced and shown no significant differance with control group(Fig.3 A). The IL-4 levels of vehicle-treated Vγ1 γδ T cells reached nearly 70pg/ml, and rapamycin-treated Vγ1 γδ T cells obviously reduced IL-4 production, which indicated that rapamycin reduced suppression via downregulating IL-4 secretion. We furthur detect some key transfactor and cytokine of rapamycin-treated Vγ1 γδ T cells. Results shows that Vγ1 γδ T cells rarely express Foxp3, and slightly up-express IFN- , suggesting these factors do not involve in suppressing function of Vγ1 γδ T cells. Based on the fact that CD4+ T cells diffrentiate into Th2 cells and then produce IL-4 under GATA-3 regulation, we detected the GATA-3 expression of Vγ1 γδ T cells. it's obvious that CD4+ T cells, Vγ1 and Vγ4 γδ T cells significantly up-express GATA-3 in Th2 condition, and remarkly reduced after rapamycin treatment, which indicated that rapamycin inhibited the GATA-3 signaling of T cells.

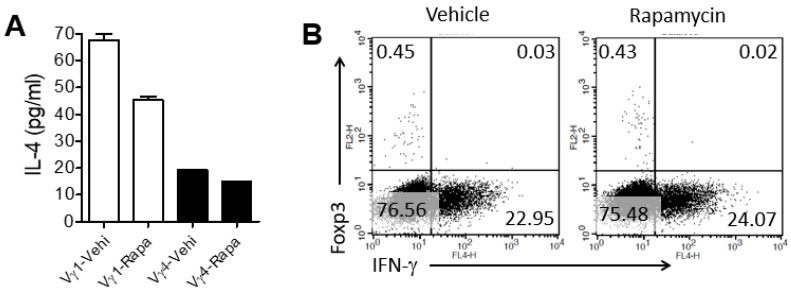

Fig. 3 Rapamycin treatment reduce IL-4 production of Vγ1 γδ T cells

A. Rapamycin treatment suppress IL-4 production of Vγ1 γδ T cells; B. Foxp3 and IFN- expression of Vγ1 γδ T cells do not altered after rapamycin treatment.

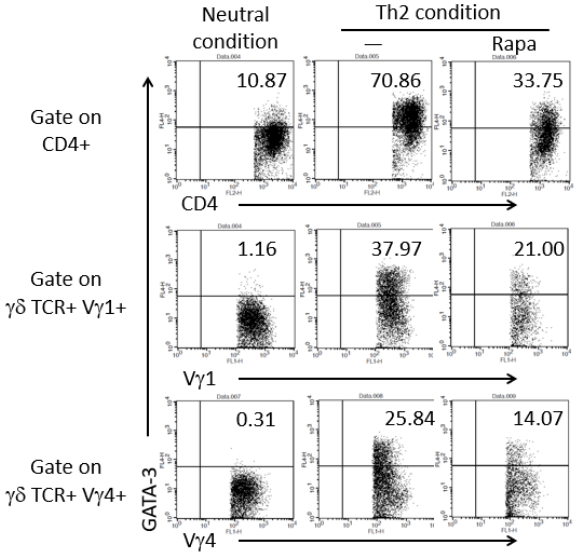

Fig.4 Rapamycin treatment suppress GATA-3 expression of Vγ1 γδ T cells

4. Conclusion and Discussion

According to our findings in this paper, we speculated rapamycin inhibited mTOR and GATK-3 signaling and downregulated IL-4 production of Vγ1 γδ T cells, finally led to the reduced suppreing

function of Vγ1 γδ T cells towards Vγ4 γδ T cells. Our research results may further improve the regulatoty network between γδ T cells and tumor therapy.

There's a study revealed that TWS119, an activator of Wnt signaling, could remarkly enhance the proliferation, differentiation and cytotoxicity of γδ T cells against tumor cells[20]. In this process, the enhanced proliferation and survival of γδ T cells is not only by activated Wnt pathway, but also by active mTOR signaling, up-expression of anti-apoptosis protein Bcl-2. This research is a differant scale of studying γδ T cells and mTOR signaling compared to our research(activation and inhibiton of mTOR signaling), which sugguest the interaction between γδ T cells and tumor cells is very complecated, and it's need to further research to provide more knowledge into tumor therapy by γδ T cells.

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