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UNDERSTANDING THE ROLE OF NZF3 IN THE IMMUNOSUPPRESSIVE EFFECTS OF ANISOMYCIN ON T CELLS

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Abstract: Anisomycin, a protein synthesis inhibitor with potential chemotherapeutic applications, has been shown to inhibit inflammatory responses and the activation of T cells, reducing its potential anti-cancer effect. NZF3, a transcription factor, on the other hand, enhances inflammation and the expression of pro-inflammatory genes. This study aimed to investigate the impact of knocking down the NZF3 gene in T cells on the immunosuppressive effects of anisomycin. The study employed a method for knocking down NZF3 using siRNA in T cells. Results indicated that the knockdown of the NZF3 gene partially reversed the immunosuppressive effects induced by anisomycin. Specifically, colony formation, CD69, CD25, and CD71 expression, and secretion of pro-inflammatory cytokines were increased. These findings suggest that NZF3 may be a potential target to optimize the use of anisomycin for cancer treatment and provide new insight into combination therapies utilizing anisomycin.

Keywords: Anisomycin, Chemotherapy, Inflammatory Responses, NZF3, siRNA, T cells

Introduction

Anisomycin, a protein synthesis inhibitor discovered in the 1950s, shows promising potential in the treatment of cancer. The drug has been shown to have notable anticancer activity in preclinical studies, particularly in hematologic malignancies [8]. However, a limitation of this drug is its tendency to inhibit inflammatory responses, including the activation of T cells, which can reduce its potential anti-cancer effect. Therefore, new strategies need to be developed to optimize the use of anisomycin in cancer treatment.

NZF3 is a transcription factor that has been shown to enhance inflammation and the expression of pro-inflammatory genes [10]. As such, it may have a role in the regulation of the immunosuppressive effects of anisomycin. Hence, the present study aimed at determining the impact of knocking down the NZF3 gene in T cells on the immunosuppressive effects of anisomycin. The study includes a method for knocking down NZF3 using siRNA in T cells.

The results of this study may provide new insights into combination therapies utilizing anisomycin that involves targeting of the NZF3 gene in T cells. Thus, the study may offer leads for the development of a more effective and personalized treatment for certain types of cancer.

2. Material and method

2.1 Materials and Animals

Anisomycin (Sigma-Aldrich, purity: >97%) was initially dissolved at a concentration of 20 mg/ml for stock in phosphate-buffered saline (PBS) at −20 ℃. Lipopolysaccharide (LPS) and Concanavalin A (Con A) were both bought from Merck/Sigma-Aldrich (St. Louis, MO, USA). **2.2 Animals**

Female C57BL/6 mice (6-8 weeks old), brought from Laboratory Animal Center of Southern Medical University (Guangzhou, China), were reared under specialized pathogen-free circumstances in accordance and in compliance with the guidelines established by the university's Animal Care Committee.

2.3 Cell separation and culture

Cervical dislocation was used to sacrifice experiment mice in order to separate their mesenteric lymph nodes under aseptic conditions. Lymph nodes were harvested, mechanically grinded and then filtered through a 40-μm cell strainer so as to obtain individual lymphocytes. Cells were resuspended in RPMI-1640 complete medium after being rinsed twice with ice-cold PBS, and then incubated at 37 ℃ in a humid atmosphere containing 5% CO_2.

2.4 Silence of NZF3

Three paired NZF3 siRNAs were used to transfect T cells. si-NZF3#1: 5'- GCAUCG GGAGUUCCAAUUATT-3' and 5'-UAAUUGGAACUCCCGAUGCTT-3'; si-NZF3#2: 5' -GCUGCCAUCCUGAACCUUUTT-3' and 5'-AAAGGUUCAGGAUGGCAGCTT-3'; si-NZF3#3: 5'-GGUCAAUGCUGCCUUCUAUTT-3' and 5'-AUAGAAGGCAGCAUUGAC CTT-3'; NC siRNAs were employed as a negative control: 5'-UUCUCCGAACGUGU CACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'.

2.5 PCR analysis

Following the instructions of manufacturer, total RNA was extracted using trizol reagent (Invitrogen, Cat no.15596-018) and RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) was used to reverse-transcribe the extracted total RNA into cDNA. The relative expression of mRNA was measured by PCR after being amplified with special primers. Primer sequences of *NZF3* were as follows: 5'-AGTCCGTGCCAGCTCTTATG-3' and 5'AGAGGATGTCTGTGGCTTCC-3'.

2.6 Flow cytometry analysis

Surface marker attaining with PE/FITC/APC-labeled mAbs were performed as described earlier[13]. In brief, splenic-derived T lymphocytes were treated with 5 ng/ml ConA and 1 μg/ml LPS or pre-transfected with NZF3 siRNA before treatment of 50 ng/ml anisomycin. FITC-conjugated CD3 and PE-conjugated CD25, CD69 or CD71 antibodies were used to stain cells from each group, and cells were then determined by flow cytometry. FlowJo v10 software was used to analyze the data.

2.7 Detection of cytokines

LPS-stimulated T cells were either treated with 50 ng/ml anisomycin alone or pre-transfected with NZF3 siRNA before anisomycin treatment. Mouse ELISA kits (DAKEWEI, Shenzhen) were used to determine the secretion of IL-6, IL-1β, TNF-α and IFN-γ in the cell-culture supernatants in each group according to the manufacturer's protocol. With the use of a microplate reader, the absorbance was determined at 450 nm. Three duplicate wells were conducted for each group.

2.8 Statistical analysis

All data were expressed as mean ± standard deviation (SD) and statistical analysis was carried out through GraphPad Prism 8 using the Student *t* test for paired and unpaired data and the oneway analysis of variance.

3. Results

3.1 Confirmation of the knockdown of *nzf3* gene

Three paired NZF3 siRNAs were used to deplete *nzf3* in T cells. They had the similar effects on T cells, but si-NZF3#2 was slightly more effective (Figure 1) and was utilized to knockdown *nzf3*.

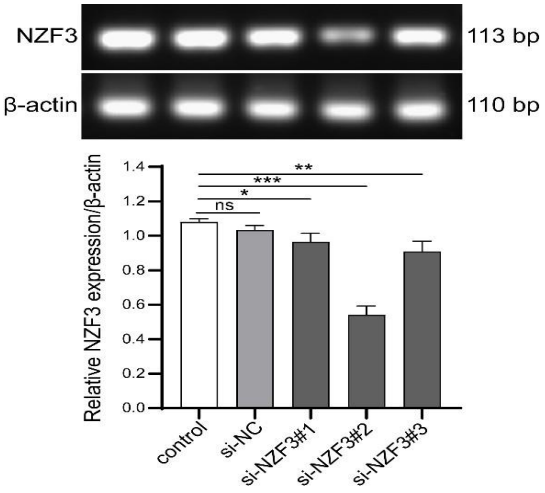

Figure 1 Expression of NZF3 in T cells treated with or without different NZF3 siRNA. T cells separated from C57BL/6 mice was transfected with three NZF3 siRNA (si-NZF3#1, siNZF3#2 or si-NZF3#3) or NC siRNA (si-NC) and the knockdown effect of *nzf3* was quantified by PCR. The results were normalized by β-actin. Data are expressed as means ± SD of three experiments. **P* < 0.05 *vs*. the control group.

3.2 **Knockdown of *nzf3* attenuates the immunosuppressive effect of anisomycin on T cells** It is reported that anisomycin significantly suppresses the activated T cells and affects inflammatory response. To determine a relationship between NZF3 and effect of anisomycin, T cells were activated with ConA or LPS to trigger inflammatory responses, and then were given anisomycin treatment in the presence or absence of NZF3 siRNA. Under an inverted microscope, we found that the knockdown of *nzf3* resulted in significant enhancement in the size and density of cell colonies which has been reduced by anisomycin (Figure 1A). CD69, CD25 and CD71 were makers of T cell activation stages[14]. According to the flow cytometry results, anisomycin decreased these T cells' activation markers, but this effect was fully reversed when *nzf3* was silenced (Figure 2B). As IL-6, IL-1β, IFN-γ and TNF-α are typical pro-inflammatory cytokines which can suppress and relieve inflammatory responses by activating local and systemic inflammatory responses, we next evaluated the secretion of these cytokines in LPS-stimulated T cells by ELISA. Likewise, *nzf3* knockdown remarkably promoted the secretion of cytokines which has been inhibited by anisomycin (Figure 2C). These findings suggest that the knockdown of *nfz3* in T cells reverses the immunosuppressive effects induced by anisomycin.

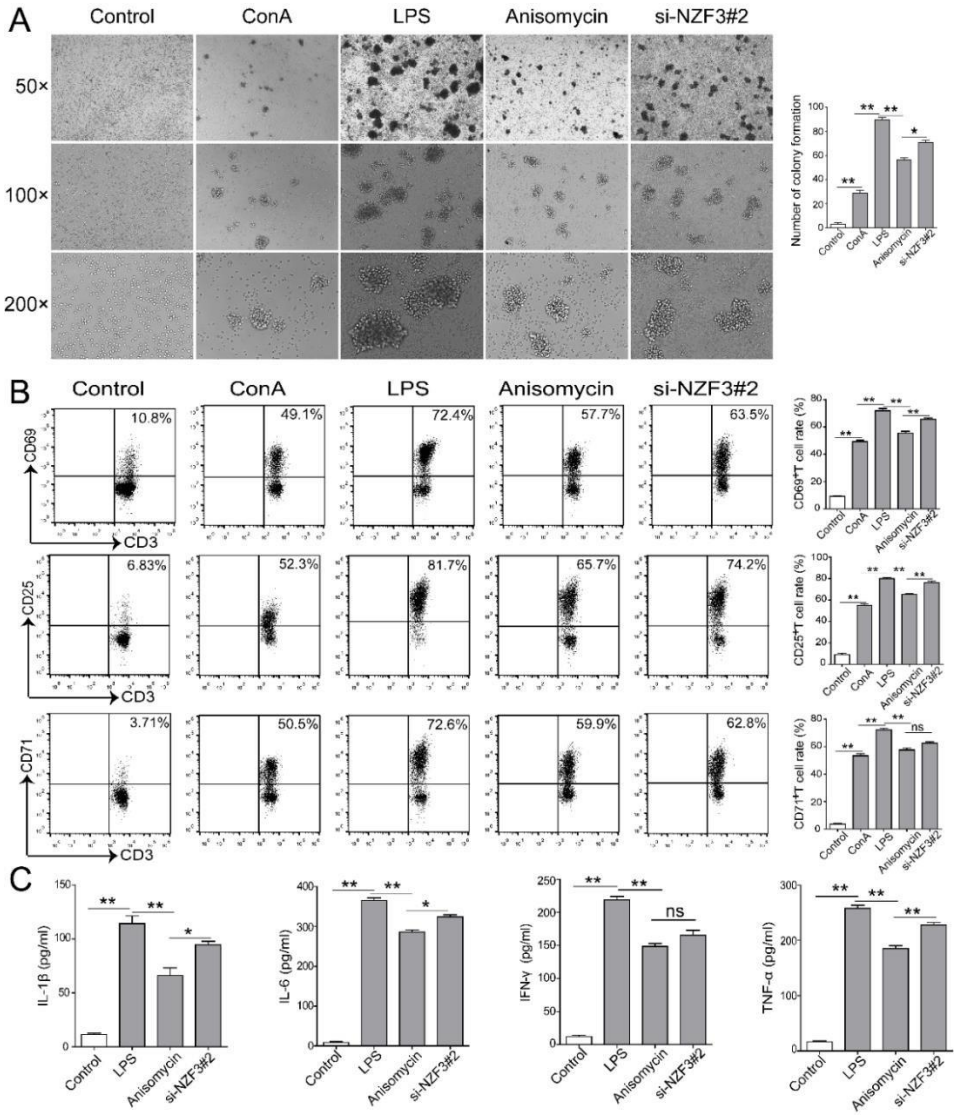

Figure 2 Effects of *nzf3* knockdown on the anisomycin-suppressed inflammatory responses in T cells. LPS-stimulated T cells were treated with or without 50 ng/ml anisomycin for 24 h in the present or absent of si-NZF3#2. The effects of the different treatment on colony formation (A), the expression of CD69, CD25 and CD71 (B), and the level of IL-6, IL-1β, IFN-γ and TNF-α (C) in LPS-stimulated T cells were examined under an inverted microscope, by flow cytometry and ELISA, respectively. Data is expressed as mean ±SD (n = 3). *P <0.05; **P <0.01; ***P <0.001; ns, not significant.

4. Conclusion

In summary, *nzf3* knockdown is successfully triggered by *nzf3* siRNA, and down-regulation of NZF3 increases the colony formation, expressions of CD69, CD25 and CD71, and secretion of IL-6, IL-1β, IFN-γ and TNF-α in T cells treated with anisomycin, which indicating that NZF3 can partially reverse the immunosuppressive effect derived from anisomycin, providing a new thinking about combination therapy of anisomycin.

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