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NZF3 MEDIATES ANISOMYCIN TO INHIBIT THE ACTIVATION AND INFLAMMATORY CYTOKINE SECRETION OF T CELLS

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Abstract: Anisomycin, a protein synthesis inhibitor, can inhibit the activation, proliferation and inflammatory responses of T cells. Emerging evidence shows that transcription factor NZF3 participates in the expressions of pro-inflammatory genes. No report is, to date, involved in whether it plays a considerable role in anisomycin-induced immunosuppression. In the current study, T cells was treated with or without anisomycin in the present or absent of NZF3 siRNA. The results indicate that the knockdown of *nfz3* gene in T cells partially reverses the immunosuppressive effects induced by anisomycin.

Keywords: Anisomycin, NZF3, biobehaviour, T cells.

1. Introduction

Anisomycin is one of antibiotics, which can inhibit protein synthesis in cells via its combining ability to ribosome[1-3]. Anisomycin was firstly isolated from *Streptomyces griseous*, and early studies revealed that it could trigger apoptosis in different cells by activating particular pathway, such as P38 and ERK1/2, making it potentially become a viable medication for chemotherapy treatment[4-6]. On the other hand, some researches also discovered that anisomycin was able to inhibit the inflammatory responses and activation of T cells with immunosuppression, which may attenuate its anti-cancer effect[7, 8]. Therefore, it is necessary to find out crucial factors to eliminate anisomycin-induced immunosuppressive effect.

Neural zinc finger 3 (NZF3), also called st18/Myt3, which was initially discovered to inhibit the development of breast cancer[9], is a transcription factor of the Neural zinc finger (NZF) protein family members, sharing distinctive CCHHC-type zinc finger motifs as DNA-binding domains. It has been reported that NZF3 increased the inflammatory effect stimulated by LPS

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for its deficiency, suggesting that it might be an important inflammatory regulatory factor[10, 11]. Furthermore, the knockdown of NFZ3 also delayed tumor progression and resulted in depletion of tumor-associated macrophages in liver cancer[12]. Taken together, NZF3 may be a novel regulator of anisomycin to bring about an optimum curative effect in anti-cancer treatment. Here, in order to investigate a relationship between NZF3 and anisomycin, NZF3 siRNA was used to knockdown of *nzf3* for revealing a potential effect of NZF3 in T cells treated with anisomycin.

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 micro-plate 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 one-way 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*.

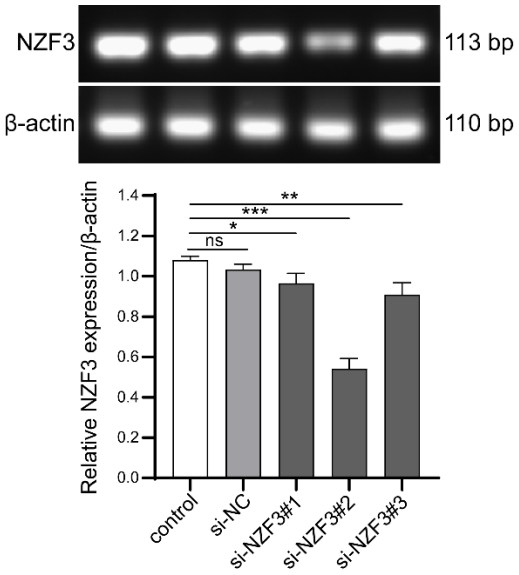

Figure 1 Expression of NZF3 in T cells treated with or without different NZF3 siRNA. T cells separated from C57BL/6 mice was transfected with three NZF3 siRNA (si-NZF3#1, si-NZF3#2 or si-NZF3#3) or NC siRNA (si-NC) and the knockdown effect of *nzf3* was quantified by PCR. The results were normalized 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.

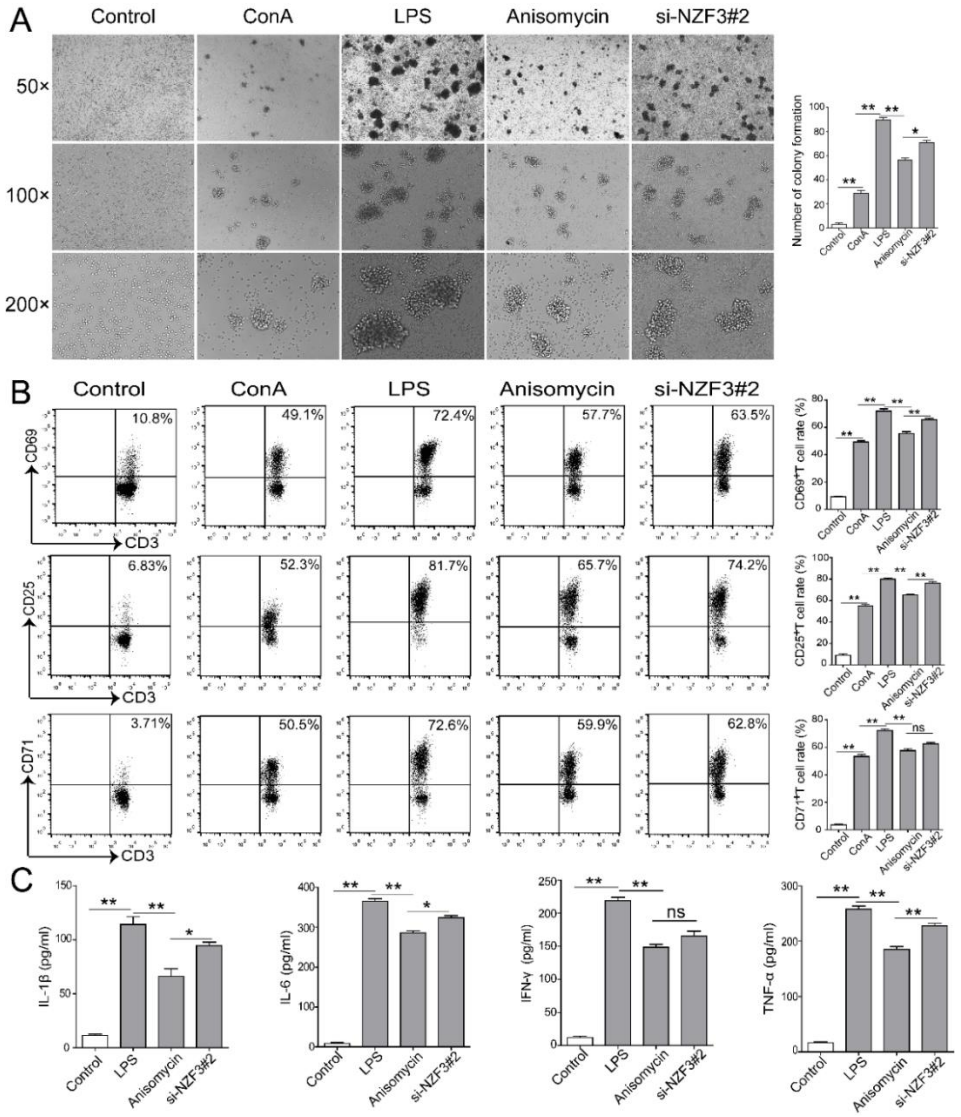

Figure 2 Effects of *nzf3* knockdown on the anisomycin-suppressed inflammatory responses in T cells. LPS-stimulated T cells were treated with or without 50 ng/ml anisomycin for 24 h in the present or absent of si-NZF3#2. The effects of the different treatment on colony formation

(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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