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SHP099 Enhances CD8+ T Cell Activity and Promotes its Memory Phenotype Formation

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Abstract

Known as intracellular checkpoint proteins (ICPs), Src-homology domain-containing protein tyrosine phosphatase-2 (SHP2) deactivates multiple immune cells through dephosphorylation of key molecules that were phosphorylated and activated by stimulating signal, eventually leading to immunosuppression. No report is, to date, involved in effects of SHP2 inhibitor SHP099 on CD8+ T cells directly in vitro. In this study, SHP099 was chosen to block SHP2 signaling, and the maximum concentration that did not damage cell viablity was selected. Human primary CD8+ T cells were treated with or without SHP099, and their viability, secretion function and memory phenotypes were examined. The results indicate that the inhibition of SHP2 signaling enhances the activity of CD8+ T cells, leading to differentiation into memory phenotypes. This provides some experimental basis for a deeper elucidation of the influence of SHP2 signaling on T-cell activation, function and memory.

Keywords

SHP099, SHP2, cytokine, memory phenotype, CD8+ T cell.

1. Introduction

T cells are important "executors" of antiviral and anti-tumor immunity in the organism, playing a vital role in adaptive cellular immune responses. Among T cells, $CD8^+$ T cells secrete various cytokines, simultaneously, directly mediate the killing of some viruses, tumor cells and the purging of other antigenic material, playing a particularly prominent effector function[1]. However, inhibitory checkpoint receptors (ICR) from T cells, such as programmed cell death protein 1 (PD-1), have been verified to astrict the activity of T cells, contributing to T cell exhaustion that ultimately brings about immunity failure[2]. Therefore, it is essential to find out effective measure to eliminate ICR-induced immunosuppression.

SHP2, one of intracellular checkpoint proteins (ICPs), is recruited by the intracellular tail of the immune checkpoint (IC) on T cells[3-6], followed by dephosphorylating the critical signaling proteins of activated TCR signaling through the Src homology region 2 (SH2) domain, ultimately leading to immunosuppression[7, 8]. It seems to draw a conclusion that SHP2 exerts a negative influence on T cells-mediated immune responses. Paradoxically, it has been reported that mouse tumor models with SHP2-deficient T cells show decreased TCR-mediated IL2 secretion[9] without enhanced tumor clearance or the synergistic effects on anti-PD-1 therapy[10]. Whereas, blocking SHP2 signaling with an allosteric inhibitor SHP099 combined with blocking PD-1 showed the enhanced immune response in mouse tumor models[11]. Taken together, SHP099 may be a novel immunostimulant for $CD8^+$ T cells. Here, for the purpose of investigating the relationship between SHP2 signaling and $CD8^+$ T cells and avoiding uncertainties that arise from genetic inactivation of SHP2 signaling, SHP099 was used to block

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SHP2 signaling in $CD8^+$ T cells seperated from human peripheral venous blood to reveal its potential effect on their viability, production of cytokines and memory phenogypes.

2. Material and method

2.1. Material

SHP099 (Boer, shanghai, purity: >99%) was initially dissolved at a concentration of 4 μM for stock in dimethyl sulfoxide (DMSO) at −20 ℃. Human primary $CD8^+$ T cell culture medium was prepared with 90% RMPI-1640 basic culture medium (Gibco, USA) and 10% human AB serum (Access Biological, USA) by addition of 1 μg human recombinant IL-2 and 0.5 μg human recombinant IL-7 (Thermo scientific, USA), and stored at 4 ℃ for a short period of time.

2.2. CD8+ T cells separation and culture

Fresh human peripheral blood was collected into the anticoagulant vessels, diluted with 0.9%NaCl solution of equal volume, stratified by Lymphoprep (Serumwerk Bernburg AG, Ger), and then the white membrane was absorbed as a monocyte layer. Cells were re-suspended as single-cell suspension by ImunoSep Buffer (Biolegend, USA). $CD8^+$ T cell sorting kit (Biolegend, USA) was used to sort $CD8^+$ T cells on immune magnetic beads under the guidance of manufacturer's protocol, and the cells were cultured in the Human primary $CD8^+$ T cell culture medium. In addition, 1 μg/mL anti-CD3 functional monoclonal antibody and anti-CD28 functional monoclonal antibody (Multisciences, Peking, China) were added.

2.3. Detection of cell viability

With the Cell Counting Kit-8 (CCK-8) assay, cell viability of suspension cultured cells was mearsured. $CD8^+$ T cells obtained from human peripheral venous blood were exposed to various concentrations of SHP099 (1, 2, 4, 8, and 16 μM) for 48 hours or 1 μM SHP099 for different periods (24, 48, and 72 hours). After the treatment, added CCK-8 reagent (Beyotime Biotechnology, Shanghai, China) to reach a final concentration of 10%. A microplate reader (Thermo scientific, USA) was used for detection of absorbance of each group at 450 nm, and each group had five duplicate wells.

2.4. Detection of cytokines

$CD8^+$ T cells were exposed to SHP099 for 48 hours. Human ELISA kits (JSBOSSEN, Jiangsu, China) were used to determine release of IL-2, IFN-γ and TNF-α in supernatants under the guidance of instructions. A microplate reader (Thermo scientific, USA) was used for detection of absorbance of each group at 450 nm, and each group had three duplicate wells.

2.5. Analysis of memory phenotypes

$CD8^+$ T cells were treated with SHP099 for 48 hours, cells from each group were stained using APC-conjugated anti-mouse/human CD44 (Elabscience, Wuhan, China) and PE-conjugated anti-human CD62L antibodies (Elabscience, Wuhan, China), and measured by flow cytometry. A FlowJo VX software was used for data analyzing.

2.6. Statistical analysis

Univariate analysis of variance (GraphPad Prism 8.3) was performed using Tukey's post-hoc test. The data represented three to five independent replicates and all original data were shown as mean ± standard deviation (SD).

ISSN: 1813-4890

3. Results

3.1. Appropriate working concentration and duration of SHP099

To explore the positive effects of SHP2 signaling inhibition on CD8+ T cells using SHP099, we need to determine the maximum concentration and duration of SHP099 that does not damage the cell viability of CD8+ T cells. First, various concentrations of SHP099 were used to treat CD8+ T cells for 48 hours. As shown in Figure 1a-b, the viability of CD8+ T cells gradually decreased with concentrations greater than 1 μM of SHP099. Hence, we selected 1μM as the working concentration of SHP099 and treated CD8+ T cells for different durations(24, 48, and 72 hours). As shown in Figure 1c-d, the cell viability of CD8+ T cells was observably decreased with the treatment of 1 μM SHP099 for 72 hours, but showed no significant alteration at 24- or 48-hour treatments. These resullts suggest that 1 μM SHP099 for 48-hour treatment would be a properly working condition for SHP099 to act on CD8+ T cells.

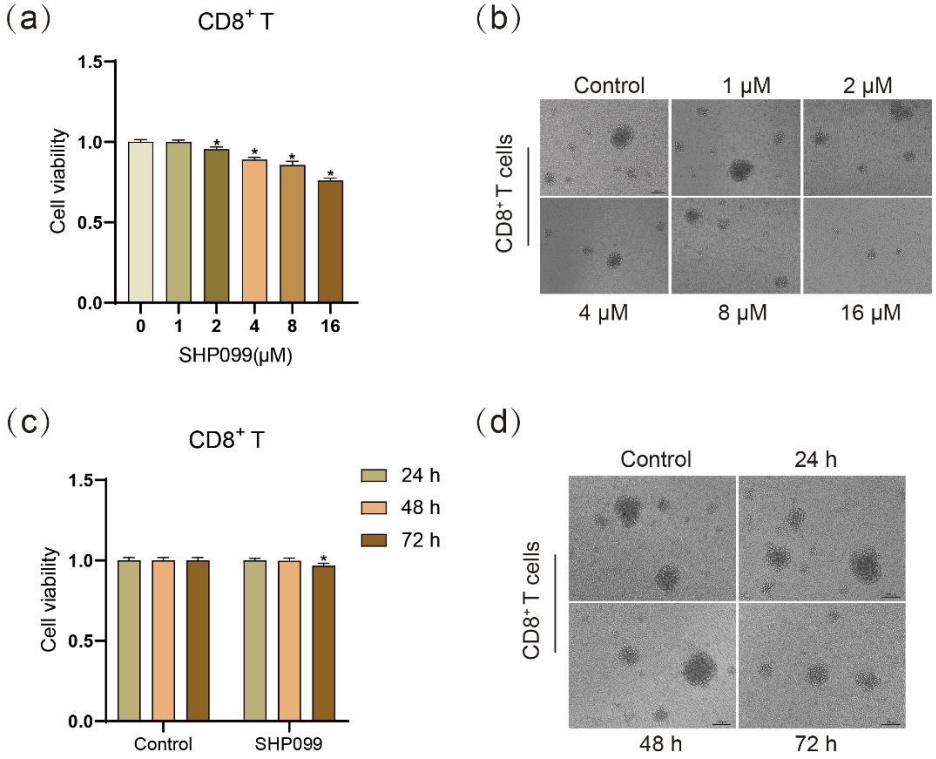

Fig. 1 Low concentration of the SHP2 inhibitor SHP099 does not affect cell viability of CD8+ T cells. SHP099 was used to treat CD8+ T cells at concentrations of 0, 1, 2, 4, 8, and 16 μM for 48 hours, respectively. The differences in cell viability between each group were analyzed by the CCK-8 assay (a) and observed under an inverted microscope (b). The CD8+ T cells were exposed to 1 μM SHP099 for 24, 48, and 72 hours, respectively, and their viabilities were analyzed by the CCK-8 assay (c) and observed under an inverted microscope (d). The data are shown as mean ±SD (n = 5), where *P <0.05 indicates statistical difference.

3.2. Low concentration of SHP099 promotes CD8+ T cells to release cytokines

Interleukin-2 (IL-2), tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) are important executors of T cells. IL-2 promotes T cells to proliferate and be activated, while IFN-γ and TNF-α are very important proinflammatory cytokines that enable T cells to exert their effector function and prevent the growth of abnormal cells, such as tumor cells or virus-infected cells. Therefore, for the sake of exploring effects of SHP099 on release of these three cytokines by CD8+ T cells, the CD8+ T cells were exposed to 1 μM SHP099 for 48 hours. Afterwards, the three cytokines in the supernatants were detected using corresponding ELISA kits. In comparison to

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the untreated group, the low concentration of SHP099 prominently increased the release of IL-2, IFN-γ and TNF-α from CD8+ T cells (Figure 2a-c).

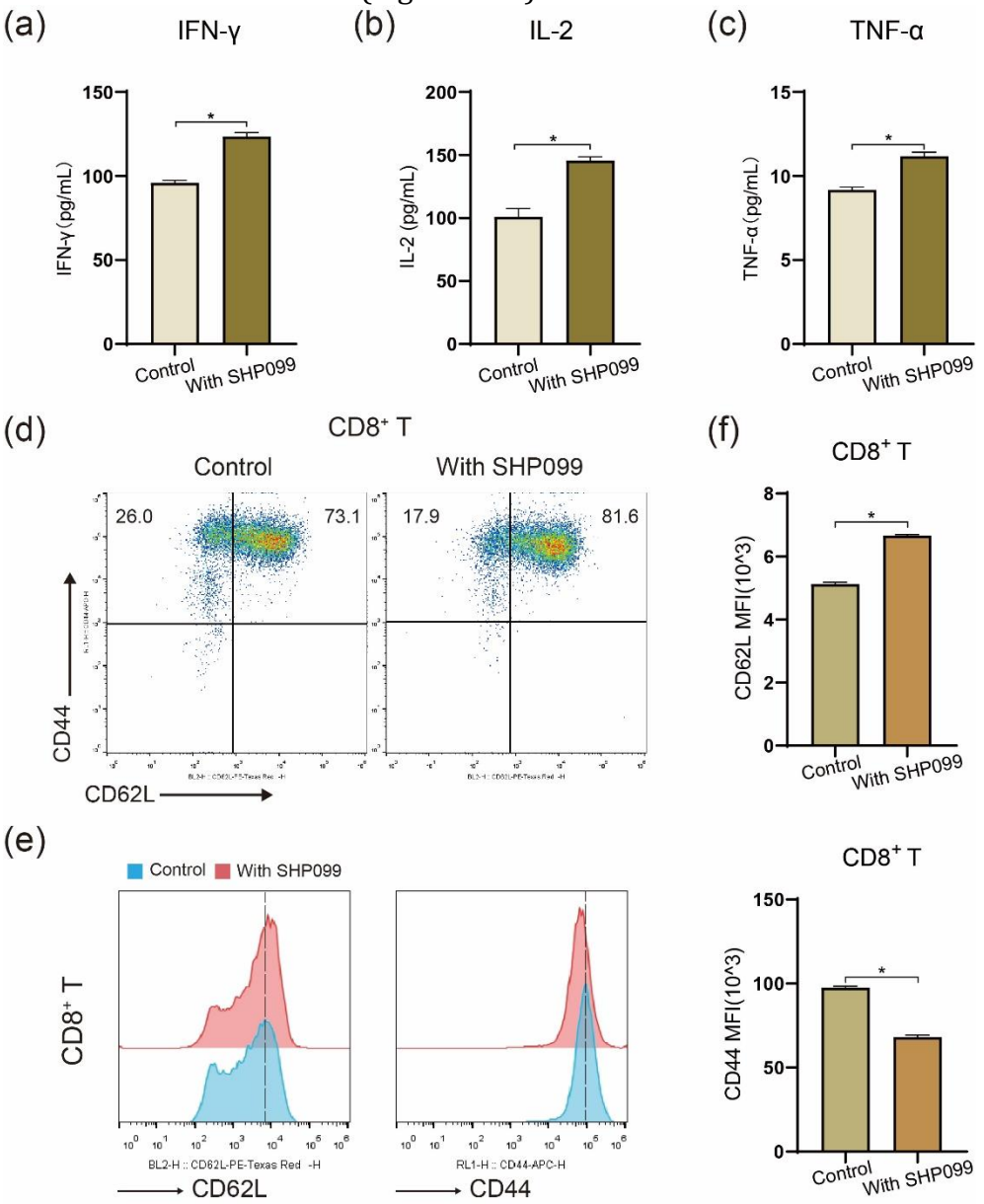

Figure 2. SHP099 drives the formation of memory phenotypes of CD8+ T cells. CD8+ T cells were exposed to 1 μM SHP099 for 48 hours, and then IFN-γ (a), IL-2 (b), and TNF-α (c) released by the CD8+ T cells were measured using the corresponding ELISA kits. The levels of CD44 and CD62L on the membrane of the CD8+ T cells were measured using APC anti-mouse/human CD44 antibody and PE anti-human CD62L antibody (d-e). The Mean Fluorescent Intensity(MFI) of each group was also determined by flow cytometry (f). The data are shown as mean ±SD (n = 3) , *P <0.05 indicates statistical difference.

3.3. Low concentration of SHP099 drives CD8+ T cells into memory phenotypes

The advantage of the memory phenotype over the effector phenotype is the reduction of T cell exhaustion, as continuous antigen exposure always results in T cell exhaustion and eventual anergy [12]. Further quantity of memory T cells tends to signify higher proliferative potential in response to

antigens-mediated re-stimulation[13, 14]. In view of this, CD8+ T cells were exposed to 1 μM SHP099 for 48 hours, then the central memory subsets of T cells (T_{CM}, CD62L+CD44+ T cells) were indicated by detecting the expression levels of the memory phenotypic biomarkers CD44

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and CD62L. As shown in Figure 2d-f, in comparison to the untreated group, the low concentration of SHP099 observably increased both the proportion of T_{CM} and Mean Fluorescent Intensity (MFI) of CD62L which tend to represent the memory biomarker on $CD8^+$ T cells.

4. Conclusion

In summary, the inhibition of SHP2 signaling in $CD8^+$ T cells by a low concentration of SHP099 can significantly enhance their ability to release proinflammatory cytokines and promote the formation of central memory subpopulation. These findings provide a novel thought about using inhibitors of downstream molecules in ICR signaling to assist $CD8^+$ T cells in exerting a stronger effect function.

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