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Short Communication

Dual function of IL-33 on proliferation of NIH-3T3 cells

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ABSTRACT

The interleukin-33 (IL-33)-ST2L signaling pathway has been shown to play important roles in the field of immunology, especially as a trigger for allergic reactions such as bronchial asthma. However, coming back to the original finding that the ST2 gene is induced during initiation of the cell cycle of fibroblastic cell lines, the possible functions of the ST2 gene products and their specific ligand, IL-33, in the field of cell growth regulation are still interesting problems to be solved.

In this study, we used NIH-3T3 mouse cell line and added IL-33 before and after cell proliferation assay, which revealed the dual function of IL-33. When IL-33 was added to the confluent cells before the start of cell proliferation, it suppressed the cell growth concentration-dependently. On the other hand, if IL-33 was added after the start of cell proliferation, it enhanced the cell growth.

The negative effect of IL-33 on cell proliferation is a novel finding and would provide an important clue to the roles of IL-33 and ST2/ST2L in growth regulation.

1. Introduction

ST2 (IL-1RL1) cDNA was cloned in the late 1980s as a gene induced in the course of cell-cycle initiation (ST2 is short for serum **ST**imulation-**2**) [1,2]. Through the subsequent cloning of cDNA for ST2L (the **L**ong form of **ST2**), which encoded a type I membrane protein resembling IL-1R [3], the main focus of the research shifted to immunological aspects. The most exciting finding was the identification of the ligand, named IL-33, which could induce various allergic reactions such as bronchial asthma [4,5].

On the other hand, the possible functions of the IL-33-ST2L system in the field of cell growth regulation are still obscure, and have been our continuous pending question. Getting back to the starting point, namely, the ST2 gene as the gene induced by growth stimulation of fibroblast, this research is focused on the functions of IL-33—which is the ligand for the receptor-type ST2 gene product, ST2L—in cell proliferation.

Here, we report intriguing dual opposing functions of IL-33 in cell proliferation.

2. Materials and methods

2.1. Cell culture

The NIH-3T3 cell line, which is able to be introduced into G_0 phase, was used [6]. Each experiment was started by thawing a new vial of cells (day −7 in Fig. 1A). After 2 days, 6×10^5 cells were seeded in a 10-cm culture dish (Corning Inc., Corning, NY) containing 10 mL of DMEM (Sigma–Aldrich Co., St. Louis, MO) supplemented with 5% (v/v) of heat-inactivated FBS (HyClone Lab., Inc., Logan, UT) (day −5 in Fig. 1A), and cultured at 36.5 °C in 5% CO_2 for 5 days. When pre-treatment with IL-33 was necessary, recombinant murine IL-33 (Peprotech Inc., Rocky Hill, NJ) was added to the 10-cm dish, resulting in 50 ng/mL, at 12 h before starting the cell proliferation assay (arrow a in Fig. 1A).

2.2. Cell proliferation assay

Based on a batch of preliminary experiments, the following assay condition was determined. The cells cultured for 5 days were treated with trypsin, and re-suspended in DMEM plus 5% FBS to 1×10^4 cells/mL. The suspension was added to each well of 24-well cell culture plates (Corning) (0.5 mL each) or to 35-mm cell culture dishes (Corning) (2 mL each) (arrow b in Fig. 1A).

Each well was supplemented with 50 μL of water soluble tetrazolium salt (WST)-1 solution (TaKaRa Bio, Otsu, Japan), and

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal bovine serum; FITC, Fluorescein isothiocyanate; IL-1R, Interleukin 1 receptor; S.D., Standard deviation.

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incubated at 36.5 °C in 5% CO_2. After 2 h, 100 μL samples were processed for measurement of the absorbance at 450 nm/620 nm. Three measurements were carried out for each well, and three independent wells were analyzed for a particular time point.

Alternatively, cells on the 35-mm dishes were treated with trypsin into 1 mL solution, and directly counted using a Sceptor™ handheld automated cell counter (Millipore Corp., Billerica, MA). Three dishes were analyzed, each in triplicate.

Every experiment was repeated at least 3 times beginning with thawing of the frozen cells.

2.3. Cell death assay

JC-1 reagent (5, 5′, 6, 6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (Cayman Chemical Company, Ann Arbor, MI) was added to 24-well plate, and incubated at 37 °C for 20 min. Then, cells were directly observed by fluorescence microscopy (Biozero-9000 (BZ-9000), Keyence, Japan). The green and red fluorescence images representing low and high mitochondrial membrane potential, respectively [7], were obtained by BZ Viewer 3.50 (Keyence). Fluorescence intensity was calculated by BZ-II Analyzer 1.31 (Keyence). The cell numbers were normalized with nuclear staining of Hoechst 33342 (Dojin, Japan). Three measurements were carried out for each well, and three independent wells were analyzed for a particular time point.

For the alternate assay, the each cell was stained with FITC-conjugated Annexin V to detect apoptosis using Annexin V index according to the manufacturer's protocol (Cayman Chemical). The fluorescence images of FITC and Hoechst 33342 were obtained by fluorescence microscopy (BZ-9000), and fluorescence intensity was analyzed and quantified by BZ-II Analyzer 1.31 (Keyence).

Five measurements were carried out for each well, and three independent wells were analyzed for a particular time point.

2.4. Immunoblot analysis

The NIH-3T3 cells introduced into G_0 phase were stimulated with DMEM plus 5% FBS during indicated periods. Then, cells were lysed with modified RIPA buffer (10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 3 mM $MgCl_2$, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.2 U/ml aprotinin, and phosphatase inhibitors), and cleared by centrifugation. Immunoblot analysis was carried out with antibodies against phosphor-ERK, phosphor-Akt, ERK, Akt, respectively. Antibodies were obtained from Cell Signaling Technology (Danvers, MA) and Santa Cruz Biotechnology (Dallas, TX).

2.5. Statistical analysis

The results were recorded as the mean ± S.D. Student's *t*-test was performed to analyze the differences in absorbance at 450 nm/620 nm, representing cell growth. A *P*-value of less than 0.01 was considered statistically significant in all experiments.

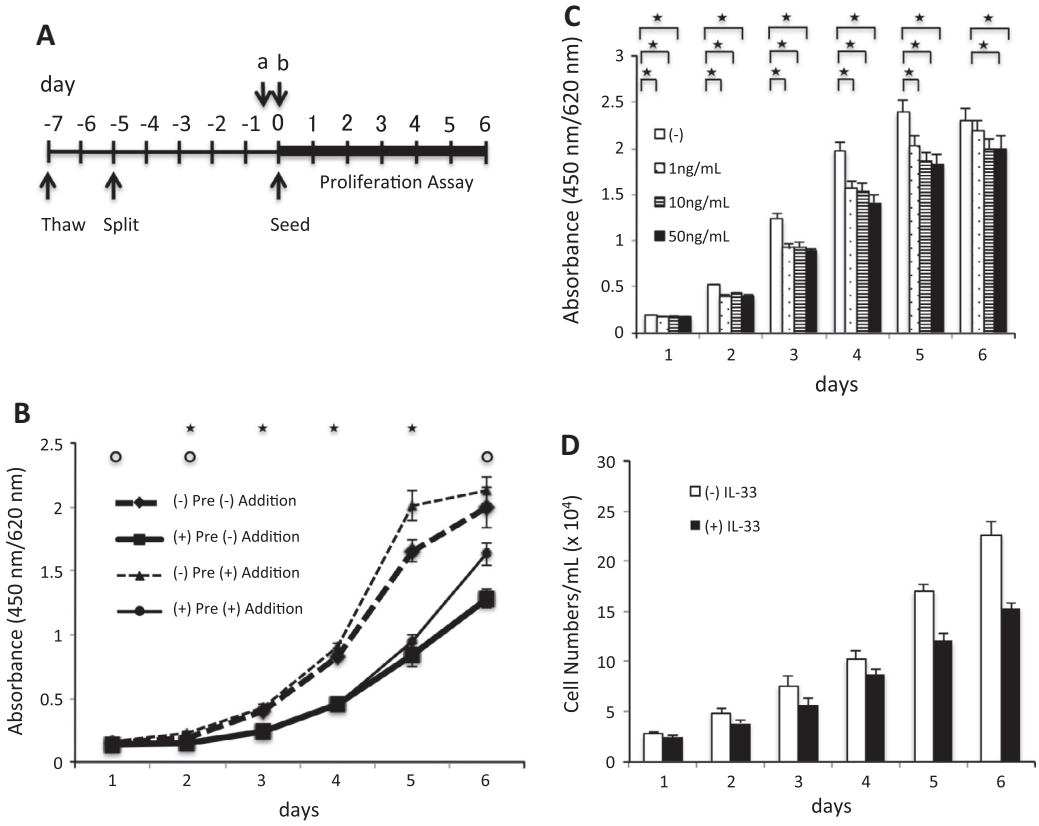

Fig. 1. Suppression of the cell growth of NIH-3T3 cells by pre-treatment with IL-33. (A) Schematic representation of the time schedule of the cell proliferation assay. Arrow a denotes the pre-treatment of density-inhibited cells with 50 ng/mL of IL-33, 12 h before starting the assay. Arrow b denotes day 0, the starting point of the cell proliferation assay. (B) A cell proliferation assay was carried out as described in the Materials and methods. Cells were pretreated (solid lines) or untreated (dotted lines) with 50 ng/mL of IL-33 (arrow a in Panel A). Cells indicated with thin lines were added with 50 ng/mL of IL-33 after starting cell proliferation. Error bars indicate the S.D. The asterisks and open circles denote *P* value < 0.01, for cells without pretreatment (dotted lines) and for cells with pretreatment (solid lines), respectively. All *P* values comparing cells with or without pretreatment were less than 10^{-8} except for those at day 1. (C) The concentration of IL-33 was changed from 1 ng/mL to 50 ng/mL for pre-treatment. Error bars indicate the S.D. The asterisks denote *P* value < 0.01. (D) Cell numbers were directly counted for IL-33-pretreated dishes (black bar) or untreated dishes (white bar) as described in the Materials and methods.