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Role of c-Myb in the regulation of natural killer cell activity

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ABSTRACT

The regulation of natural killer (NK) cell activity is an important research goal for the development of immunotherapies. In this study, we identified transcription factors affecting NK cell activity. In particular, we screened transcription factors affected by interleukin-2 (IL-2) and transforming growth factor-beta (TGF-β) by protein/DNA arrays using primary NK cells. We found that celastrol, a c-Myb inhibitor, inhibited NK-92 cells more strongly than any other inhibitors of transcription factor candidates. In addition, c-Myb and c-Myb-related signaling molecules, e.g., Nemo-like kinase (NLK) and c-Myc, were regulated by the activation status of NK cells, suggesting that c-Myb is a key regulator of NK cell activity. We also found that celastrol inhibits NK-92-cell-mediated cytotoxicity via the downregulation of NKG2D and granzyme B. Knockdown studies also showed that c-Myb is important for NK cell activation. In particular, the knockdown of c-Myb did not significantly affect NK cell proliferation and survival but decreased the secretion of IFN-γ and the cytotoxicity of NK cells. Our data demonstrate that c-Myb plays a critical role in the activation of NK cells and therefore is a therapeutic target for cancer and viral diseases.

1. Introduction

Natural killer (NK) cells can kill tumor cells and virus-infected cells, but the cytolytic activity of NK cells is dependent on their microenvironment [1]. Complex interactions among tumor cells, soluble components, stromal cells, and resident or recruited immune cells diminish the potential for an immediate inflammatory response of NK cells against malignancies [2]. The immunosuppressive tumor microenvironment must be overcome in order to use NK cells in tumor immunotherapy. To manipulate or restore NK cell activity, the identification of key factors that regulate these activities is an important goal.

Transforming growth factor-beta (TGF-β) is a major immunosuppressive cytokine in NK cells [3,4]. TGF-β suppresses the cytolytic activity of NK cells via the downregulation of NKp30, NKG2D [5], and IFN-α receptors on NK cells [6]. In addition to its role in the inhibition of NK cell cytolytic activity, TGF-β inhibits proliferation and IFN-γ secretion in NK cells, even in the presence of IL-2 [7,8].

However, the molecular mechanisms underlying the effects of TGF-β in NK cells are still being determined. TGF-β inhibits IFN-γ secretion via SMAD binding to the *Tbx21* promoter [9]. TGF-β also represses the mTOR pathway, resulting in the inhibition of NK cell activation [10]. We previously reported that TGF-β suppresses the binding activity of many IL-2-induced transcription factors, such as AR, AP-1, c-Myb, CREB, and STAT-5, in NK-92 cells [11]. TGF-β also downregulates Syk tyrosine phosphorylation and c-Myc expression in NK cells [11].

In this study, our aim was to identify a key transcription factor affected by IL-2 and TGF-β simultaneously and to evaluate its ability to determine the activation status of NK cells. We demonstrated that c-Myb is a good target for manipulating NK cell activity. Among our transcription factor candidates, the DNA binding activity of c-Myb was most highly affected by TGF-β in the presence of IL-2. We also found that celastrol, a c-Myb inhibitor, inhibited NK cell activity, even at a low dose. Furthermore, we found that the knockdown of c-Myb significantly inhibits NK cell-mediated cytotoxicity and IFN-γ secretion. These results provide a potential target for the manipulation of NK cell activity in tumor immunotherapies.

2. Materials and methods

2.1. Human NK cell preparation

Primary human NK cells were isolated and purified from the

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peripheral blood of healthy donors by negative selection using Rosettesep NK Enrichment Antibody Cocktail (StemCell Technologies, Vancouver, B.C., Canada). Primary human NK cells were purified according to the manufacturer's instructions. The freshly isolated primary human NK cells were used immediately.

2.2. Cell culture and reagent

The human NK cell line NK-92 was purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA). NK-92 cells were maintained in Minimum essential medium α (MEMα; Gibco, New York, NY, USA) supplemented with 12.5% heat-inactivated FBS, 12.5% heat-inactivated horse serum (Gibco), 0.2 mM Myo-inositol (Sigma Aldrich, St. Louis, MO, USA), 0.1 mM 2-mercaptoethanol (Sigma Aldrich), 0.02 mM folic acid (Sigma Aldrich), 1% penicillin/streptomycin (WelGENE, Gyeongsan, Korea), and 5 ng/ml recombinant human IL-2 (ATgen, Seongnam, Korea). NK-92 cells were subcultured every 2 or 3 days depending on the cell density. For some experiments, NK-92 cells were starved in IL-2-free media for 24 h. The chronic myelogenous leukemia cell line K562 and the acute T cell leukemia cell line Jurkat were purchased from the ATCC. K562 and Jurkat cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (WelGENE). The HEK 293T cell line was purchased from the ATCC and maintained in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (WelGENE). In some culture experiments, celastrol (Sigma Aldrich) was used. To stimulate human primary NK cells, IL-2 (2 ng/ml) with or without TGF-β (1 ng/ml; Cell Signaling, Danvers, MA, USA) was used.

2.3. Nuclear extraction and protein/DNA arrays

The Protein/DNA Array Kit was purchased from Panomics (Fremont, CA, USA) and used to assess the binding activity of various transcription factors. Nuclear extracts of human primary NK cells were prepared using the Nuclear Extraction Kit (Panomics) according to manufacturer's instructions. Then, 5 μg of nuclear extract was mixed with the DNA probe (10 ng/array), and each array was performed according to the manufacturer's instructions. The arrays were enhanced with chemiluminescence reagents and exposed to X-ray film.

2.4. Cell proliferation assay

After the incubation of cells (1×10^4 cells), 10 μl of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well. After 4 h of incubation, absorbance at 450 nm was measured using a microplate reader (Biotek, Winooski, VT, USA). To eliminate the background signal caused by turbidity, absorbance at 600 nm was subtracted from each well.

2.5. Flow cytometry

Primary human NK cells and NK-92 cells (~1×10^6 cells) were harvested and washed twice with cold PBS containing 2% FBS. The cells were incubated on ice with fluorochrome-labeled antibodies for 20 min. After incubation, cells were washed twice with cold PBS and resuspended in PBS containing 2% FBS and 1% paraformaldehyde for later analysis. For intracellular staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, San Diego, CA, USA). The fluorescently labeled antibodies were as follows: PE anti-human CD337 antibody (NKp30; BioLegend, San Diego, CA, USA), PE anti-human CD336 antibody (NKp44; BioLegend), PE anti-human CD335 antibody (NKp46; BioLegend), PE anti-human CD314 antibody (NKG2D; BioLegend), PE anti-human Perforin antibody (BioLegend), FITC anti-human/mouse Granzyme B Recombinant antibody (BioLegend), c-Myb Rabbit mAB (PE conjugate; Cell Signaling Technology), APC anti-human CD56 antibody (BD Biosciences), PE Isotype control antibody (BioLegend), FITC Isotype control antibody (BioLegend), and APC Isotype control antibody (BD Biosciences). For the apoptosis assay, cells were harvested and resuspended in staining buffer with the appropriate amount of Annexin V-FITC antibody and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit (BioVision, Milpitas, CA, USA). Flow cytometry was performed using the FACS LSR II (Becton-Dickinson, Franklin Lakes, NJ, USA). Data were analyzed using FlowJo software (Tree Star).

2.6. NK cell cytotoxicity assay

NK-92 cells were harvested and cocultured with calcein-AM (Invitrogen, Carlsbad CA, USA)-labeled target cells for 4 h. Calcein, the hydrolyzed form of calcein-AM, released from lysed target cells was measured using a spectrofluorometer (PerkinElmer, Waltham, MA, USA). Specific lysis was calculated as a percentage according to the following formula:

(Experimental release - Spontaneous release)/(Maximum release - Spontaneous release) × 100.

Spontaneous release was measured from the calcein released from only calcein-labeled target cells in complete growth medium. To measure maximum release, calcein-labeled target cells were incubated in complete growth medium containing 1% Triton X-100.

2.7. IFN-γ enzyme-linked immunosorbent assay (IFN-γ ELISA)

In total, 1×10^5 of NK-92 cells were treated with the appropriate amount of chemicals or cytokines for the purposes of the experiments. Culture supernatants were harvested and stored at −80 °C. IFN-γ was quantitated using the IFN-γ ELISA Kit (BD Biosciences) according to the manufacturer's instructions.

2.8. Lentiviral transduction

MYB shRNA plasmids and the control shRNA plasmid were purchased from Sigma Aldrich. Lentivirus particles were produced using a third-generation packaging system. For this system, pMDLg/pRRE (plasmid #12251; Addgene, Cambridge, MA, USA), pRSV-Rev (plasmid #12253; Addgene) and pMD2.G (plasmid #12259; Addgene) were kindly provided by Dr. Didier Trono. To transfect the plasmids into HEK293T cells, Lipofectamine 3000 was used (Invitrogen). For lentiviral transduction, NK-92 cells were stimulated with IL-2 (5 ng/ml) for 1 h and then infected by mixing with the supernatants containing lentiviral particles and protamine sulfate (15 μg/ml; Sigma Aldrich). To increase the lentiviral transduction efficiency, the mixtures were centrifuged at 360 × *g* for 90 min at 32 °C. For the selection of transgene-positive cells, the cells were cultured in complete growth medium containing 2 μg/ml puromycin for up to 4 weeks.

2.9. Real-time PCR

Total RNAs were extracted from NK-92 cells using TRIzol (Invitrogen) according to the manufacturer's instructions. A total of 1 μg of total RNA was reverse-transcribed into cDNA using a cDNA Synthesis Kit (Takara Bio Inc., Nojihigashi, Japan). Real-time PCR was performed using a thermal cycler (Applied Biosystems,

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