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

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

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This certifies that the Master's thesis of
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2018 년 여름, 신희욱 올림

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ABBREVIATION

AP-1/2	**Activator protein-1/2**
CBF	**CCAAT-binding factor**
CCK-8	**cell counting kit-8**
CD	**cluster of differentiation**
c-Myb	**avian myeloblastosis virus oncogene cellular homolog**
ELISA	**enzyme-linked immunosorbent assay**
ER	**estrogen receptor**
FAST-1	**forkhead activin signal transducer-1**
IFN-γ	**interferon-gamma**
IL-2	**interleukin-2**
MEF-1	**myeloid elf-1-like factor**
NK cells	**natural killer cells**
PAX-5	**paired box protein-5**
RAR/DR-5	**retinoic acid recptor/direct repeat-5**
STAT-4	**signal transducer and activator of transcription-4**
TF	**transcription factor**
TGF-β	**transforming growth factor-beta**

ABSTRACT

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(Directed by Professor Jongsun Kim)

Natural killer cells (NK cells) have important roles in tumor immunity. Since the status of NK cell activation is important criteria for cancer prognosis, it is an important goal for discovering factors that enhance NK cell activities. Several cytokines, such as type I interferon (interferon-α, interferon-β), IL-2, IL-12, IL-15 and IL-18, are well known to activate NK cells. Meanwhile, NK cells have been reported that their activities are inhibited by many factors in tumor microenvironment. Transforming growth factor-beta (TGF-β), one of the soluble factors in tumor microenvironment, has been studied as a potent inhibitory cytokine of NK cells. In this study, we postulated that factors which affect NK cell activity should exist and be affected by interleukin-2 (IL-2) or TGF-β, and that some transcription factors (TFs) would be such factors. We screened the transcription factors affected by TGF-β and IL-2 through protein/DNA arrays of primary NK cells. 12 transcription factors were selected and their feasibility was verified by inhibitor treatment studies. We found that celastol, a c-Myb inhibitor, potently inhibited NK-92 cells than any other inhibitors of transcription factor

candidates. In addition, c-Myb and c-Myb-related signaling molecules, such as NLK and c-Myc, appeared to be regulated by activation status of NK cells, suggesting that c-Myb is a key regulator of NK cell activity. We also found that celastol inhibits NK-92 cell mediated cytotoxicity through downregulation of NKG2D and granzyme B. Knockdown studies also showed that c-Myb is important for NK cell activation. It appeared that knockdown of c-Myb by shRNA did not significantly affect NK cell proliferation and survival, but it 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 c-Myb could be an important therapeutic target for cancer and chronic inflammatory diseases.

Key Words : natural killer cells, immunosuppression, tumor microenvironment, transforming growth factor-beta, interleukin-2, c-Myb, celastrol

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I. INTRODUCTION

Natural killer (NK) cells play a critical role in innate immunity. As their name intimates, natural killer cells can kill abnormal cells such as cancer cells, virus-infected cells and foreign cells without preceding sensitization.[1] Recently, their intrinsic killing mechanism has been described more clearly. Natural killer cells recognize their target cells through an balanced input from major histocompatibility complex (MHC) class I-specific inhibitory receptors[2] and several activating receptors that recognize ligands on target cells.[3,4] After their recognition, Natural killer cells induce target cell death by releasing cytotoxic granules including perforin[5], granzymes and granulysin[5,6], into the immunological synaptic clefts.[7] Natural killer cells also have alternative killing mechanisms by inducing apoptosis through ligands of death receptors on target cells, such as tumor necrosis factor (TNF), fas ligand (FasL, CD178), and tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL).[5] Furthermore, Natural killer cells systemically participate in innate and adaptive immune responses in host by secreting cytokines and chemokines including interferon-gamma (IFN-γ), interleukin-6 (IL-6),

granulocyte-macrophage colony-stimulating factor (GM-CSF) and so on.[8,9]

NK cells have powerful cytotoxic activity against tumor cells but NK cell activity is dependent on their microenvironment.[10] However, complicated interactions occurring among tumor cells, soluble components, stromal cells, and resident or recruited immune cells diminish the possibility of immediate inflammatory response of natural killer cells against malignancies.[11] Therefore, it is necessary to overcome the immunosuppressive tumor microenvironment to use NK cells in tumor immunotherapy. In fact, NK cells are recently more studied in the field of anti-cancer immunotherapy, because these cells have an advantage of independence on antigen specificity.[11] So, to manipulate or restore NK cell activities, finding key factors of NK cell activities is an important goal of the treatment not of alone cancer but human diseases.

TGF-β has been studied well in their role as a major immunosuppressive cytokine of NK cells.[12,13] TGF-β suppresses cytolytic activity of NK cells through downregulation of NKp30, NKG2D[14] and IFN-α receptors on NK cells.[15] In addition to its role in inhibition of NK cell cytotoxic activity, TGF-β inhibits proliferation and IFN-γ secretion of NK cells even in the presence of IL-2.[16,17]

Although many studies have been done, TGF-β is still an attractive target in the aspect of molecular mechanism studies on NK cells. TGF-β inhibited IFN-γ secretion through SMAD molecules binding to *Tbx21* promoter.[18] And also, TGF-β repressed the mTOR pathway, resulting in inhibited activation and functions of NK cells.[19] Previously, our laboratory reported that TGF-β suppressed many IL-2-induced transcription factor binding activities of AR, AP-1, c-Myb, CREB and STAT-5 in NK-92 cells.[20] And interestingly, TGF-β also downregulated Syk tyrosine phosphorylation and c-Myc expression.[20] But these evidences still have room for explanation of molecular mechanisms of TGF-β in NK cells.

In view of this situation, we aimed on finding a key transcription factor affected by

IL-2 and TGF-β simultaneously. The factor that determines activation status of NK cells could be an attractive target in manipulating NK cell activities, considering usage of NK cells in immunotherapies.

Here, we demonstrate that c-Myb, a transcription factor, is a good target upon manipulating NK cell activities. We found that binding activity of c-Myb was most affected by TGF-β in the presence of IL-2 among transcription factor candidates. We also found that celastrol, known as a c-Myb inhibitor, inhibited natural killer cell activities at low-dose. Furthermore, we found that knockdown of c-Myb significantly inhibited NK cell mediated cytotoxicity and IFN-γ secretion.

II. MATERIALS AND METHODS

1. Human NK cell preparation

Human NK cells were isolated and purified from the whole blood of healthy donors by negative selection using Rosettesep^{TM} NK enrichment antibody cocktail (StemCell Technologies, Vancouver, B.C., Canada). 50 μl of the antibody cocktail per 1 ml of whole fresh blood were mixed and incubated for 20 minutes at room temperature. The blood samples were diluted with the same volume of PBS containing 2% FBS. The diluted blood samples were placed onto a Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 20 minutes at 1200 ×g at room temperature. The purified NK cells were washed twice with PBS containing 2% FBS. The freshly isolated human NK cells were used immediately.

2. Cell culture and reagent

The human NK cell line, NK-92, was purchased from ATCC (American Type Culture Collection). 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), 0.1 mM 2-mercaptoethanol (Sigma Aldrich), 0.02 mM folic acid (Sigma Aldrich), 1% penicillin/streptomycin (WelGENE, Korea) and 5 ng/ml of recombinant human Interleukin-2 (IL-2, ATgen, Sungnamsi, Korea). NK-92 cells were subcultured every 2 or 3 days dependent on cell density. For some experiments, NK-92 cells were starved in IL-2-free media for 24 hours.

The chronic myelogenous leukemia cell line, K562, and the acute T cell leukemia cell line, Jurkat, were purchased from ATCC. K562 and Jurkat cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco) and 1%

penicillin/streptomycin (WelGENE). HEK 293T cell line was purchased from ATCC, and maintained in Dulbecco modified eagle medium (DMEM, Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (WelGENE).

In some culture experiments, we used rapamycin (1 nM to 1 μM, Sigma Aldrich), celastrol (1 nM to 1 μM, Sigma Aldrich), SIS3 (1 nM to 1 μM, Sigma Aldrich), SP600125 (1 nM to 1 μM, Enzo Lifesciences), Lisofylline (1 nM to 1 μM, Sigma Aldrich), c646 (1 nM to 1 μM, Sigma Aldrich), PD98059 (1 nM to 1 μM, Enzo Lifesciences), HLM006474 (1 nM to 1 μM, Sigma Aldrich), or 10058-F4 (1 nM to 1 μM, Sigma Aldrich) for inhibition of each transcription factor. To stimulate human primary NK cells, we used IL-2 (2 ng/ml) with or without TGF-β (1 ng/ml, Cell signaling, Danvers, MA, USA).

3. Nuclear extraction and Protein/DNA array

Protein/DNA array kit was purchased from Panomics (Fremont, CA, USA). Protein/DNA arrays were performed to assess binding activities of multiple transcription factors. To perform protein/DNA array, nuclear extracts of human primary NK cells were prepared with the nuclear extraction kit (Panomics, Fremont, CA, USA) according to manufacturer's manual. 5 μg of nuclear extracts was mixed with DNA probe (10 ng/array), and each array was performed according to the procedures in the Protein/DNA array kit manual. The Arrays were enhanced with chemiluminescence reagents and exposed to X-ray film.

4. Cell proliferation assay

After each incubations of cells (1×10^4 cells), 10 μl of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well. After 4 hour incubation, the absorbance at 450 nm was measured using a microplate reader. To eliminate the

background that comes from turbidity, the absorbance at 600 nm of each wells was subtracted respectively.

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 the ice with fluorochrome-labeled antibodies for 20 minutes. After incubation, cells were washed twice with cold PBS and resuspend in PBS containing 2% FBS and 1% paraformaldehyde for later analysis. For intracellular staining, cells were fixed and permeabilized with BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD biosciences, San Diego, CA, USA). The cells were stained with fluorochrome-labeled antibodies for 20 minutes on melting ice. After staining, cells were washed and resuspended in BD Perm/Wash™ buffer (BD biosciences, San Diego, CA, USA) for later analysis. The used fluorescent labeled antibodies are as follows : PE anti-human CD337 antibody (NKp30, Biolegend), 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 bioscience), PE Isotype control antibody (Biolegend), FITC Isotype control antibody (Biolegend), APC Isotype control antibody (BD bioscience).

For apoptosis assay, cells were harvested and resuspended in staining buffer with the appropriate amount of Annexin V-FITC antibody and propidium iodide (PI) using Annexin V-FITC Apoptosis detection Kit (BioVision). Stained cells were used immediately for apoptosis analysis. Flow cytometric analysis was performed on FACS LSR II (Becton-Dickinson). Data were analyzed with Flowjo software (Tree star).

6. NK cell cytotoxicity assays

To assess NK cell-mediated cytotoxicity, all the target cells were incubated with 1 μM calcein-AM (non-fluorescent acetomethoxy derivate of calcein, Invitrogen) for 20 minutes. After incubation, the target cells were washed and seeded into a 96-well round-bottom plate. NK-92 cells were harvested and cocultured with target cells at E/T ratios ranging from 1:1 to 5:1 for 4 hours. The calcein, hydrolyzed form of calcein-AM, released from lysed target cells was measured with a spectrofluorometer (excitation filter ; 485 nm, emission filter ; 535 nm). The percentage of specific lysis was calculated according to the following formula :

(Experimental release - Spontaneous release) /

(Maximum release - Spontaneous release) × 100

The spontaneous release was measured from the calcein released from only calcein-labeled target cells in complete growth medium. For the measurement of maximum release, calcein-labeled target cells were incubated in complete growth medium containing 1% triton X-100.

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

NK-92 cells (1×10^5 cells) were treated with appropriate amount of chemicals or cytokines for the purpose of experiments respectively. The supernatants were harvested and stored at -80 ˚C. The amount of IFN-γ was quantitated by using IFN- γ ELISA set (BD bioscience) according to the manufacturer's instructions.

8. Lentiviral transduction

To package lentiviruses, MYB shRNA plasmids and control shRNA plasmid were

purchased from Sigma Aldrich. Lentivirus particles were produced by using a third-generation packaging system. For this system, pMDLg/pRRE (Addgene plasmid #12251), pRSV-Rev (Addgene plasmid #12253) and pMD2.G (Addgene plasmid #12259) were kindly gifted from Dr. Didier Trono. To transfect the plasmids, we used Lipofectamine3000 (Invitrogen). The supernatant containing virus particles was harvested at 24 hour and 52 hour post-transfection. To eliminate cell debris, the supernatant was centrifuged at 2000 ×g and filtered through a 0.45-μm filter. The cell-free supernatant was aliquoted and stored at -80℃ for later use. For lentiviral transduction, NK-92 cells were stimulated with IL-2 (5 ng/ml) for an hour, and then infected by mixing with the supernatants containing lentiviral particles and protamine sulfate (15 μg/ml, Sigma Aldrich). To increase lentiviral transduction efficiency, the mixtures were centrifuged at 360 ×g for 90 minutes at 32℃. For selection of transgene-positive cells, the cells were cultured in complete growth medium containing 2 μg/ml puromycin for up to 4 weeks and used for analysis.

9. Real-time PCR

To assess gene expression level, total RNAs were extracted from NK-92 cells by using TRIzol (Invitrogen) according to the manufacturer's instructions. 1 μg of total RNAs were reverse-transcribed into cDNA using cDNA synthesis Kit (Takara Bio Inc., Nojihigashi, Japan). Real-time PCR was performed with Thermal Cycler (Applied biosystems, Foster city, CA, USA) and SYBR™ Green master mix (Roche, Basel, Switzerland). The data were normalized to the amount of GAPDH transcript. The primer sequences were as follows ; 5'-catgttccataccctgtagcg-3' and 5'-ttctcggttgacattaggagc-3' for c-MYB, and 5'-cagcctcaagatcatcagca-3' and 5'-gtcttctgggtggcagtgat-3' for GAPDH.

10. Immunoblotting

To assess protein expression level, 1×10^6 NK-92 cells were harvested and washed with cold PBS. To extract total proteins, the pelleted cells were lysed in Pierce™ RIPA buffer (Thermo Scientific) containing Halt™ Protease&Phosphatase Inhibitor Cocktail (Thermo Fisher). After lysis of the cells, cell lysates were centrifuged at 14000 ×g for 15 minutes at 4℃. The supernatants of cell lysates were mixed with sample buffer and boiled for 5 minutes. Total 30 μg proteins of each samples were used for immunoblot assays. The used antibodies were as follows : c-Myb (Cell Signaling Technology, Danvers, MA, USA), c-Myc (Santa Cruz, CA, USA), α-tubulin (Santa Cruz), NLK (Cell Signaling Technology).

11. Statistical analysis

Comparisons between samples were analyzed for statistical significance by using the Student t Test with Microsoft Excel software. A P value of less than .05 was considered significant.

III. RESULTS

1. TGF-β inhibits multiple transcription factor DNA binding activities in human primary NK cells

To screen transcription factors affecting human primary NK cell activity, we performed transcription factor (TF) binding activity assay by using DNA/protein array (Figure 1A). 2 ng/ml of IL-2 was used as an activator of human NK cells, whereas 1 ng/ml of TGF-β1 was used as an inhibitor of human NK cells. As shown in Figure 1a, IL-2 increased the DNA-binding activities of many TFs, while TGF-β decreased their activities. Spot intensity of each TFs was plotted as relative intensity fold changes shown as to those in cells treated with IL-2 alone (Figure 1B). 25 TFs appeared to be significantly down-regulated by TGF-β compared with those of IL-2 treated.

To quantitatively analyze the TF binding activities, we scored relative fold changes of each transcription factor binding activity and plotted in reversed bi-axis X-Y graph (Figure 1C). Using this analysis, we narrowed down the 25 TF candidates to 12 TFs for further analysis (Figure 1D). These 12 TFs which are more affected by IL-2 and TGF-β include CBF, c-Myb, AP-1, ER, FAST-1, AP-2, PAX-5, RAR/DR-5, SMAD-3/4, STAT-4, and MEF-1.

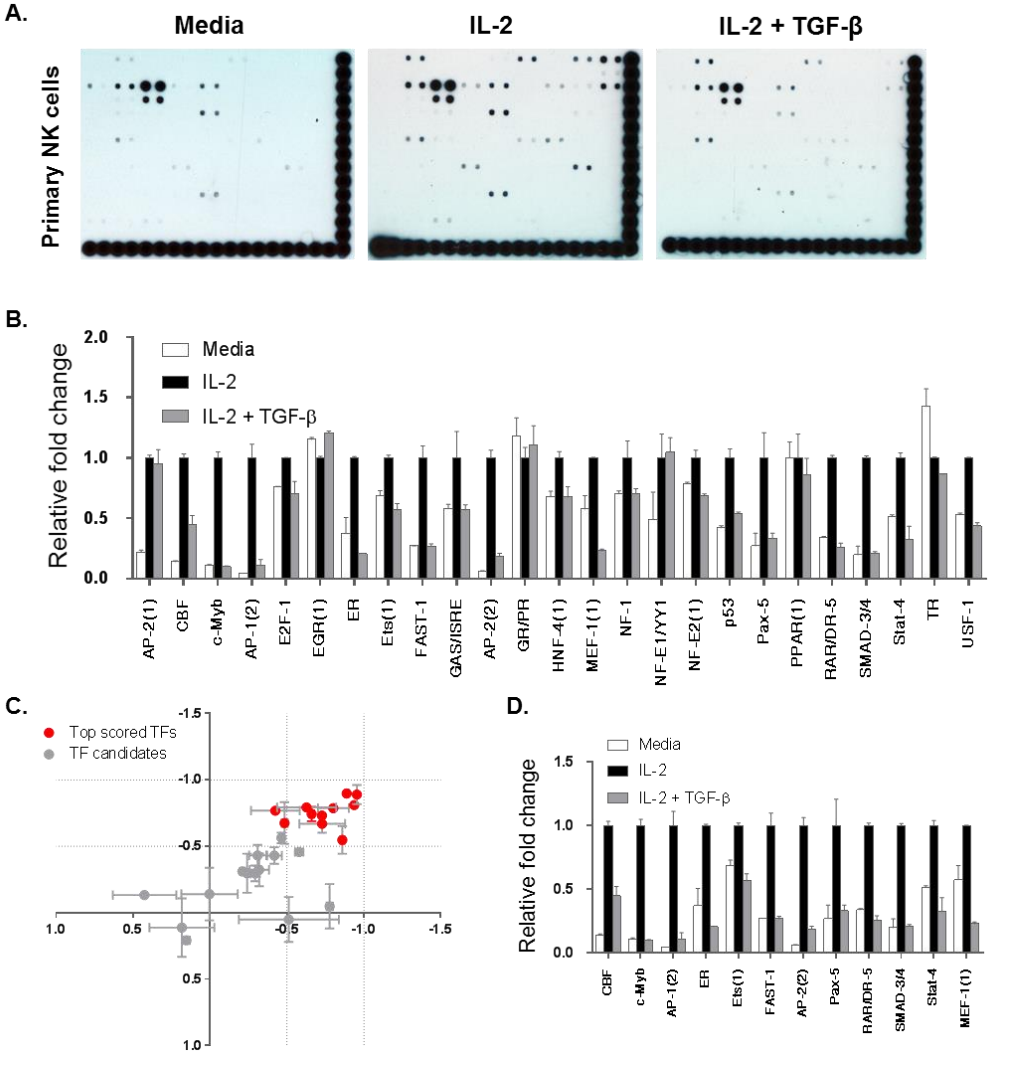


Figure 1. Changes of transcription factor binding activity in human primary NK cells by IL-2 and TGF-β. (A) To assess the changes of transcription factor binding activity in human primary NK cells, we treated IL-2 (2 ng/ml) with or without TGF-β (1 ng/ml), and performed DNA/protein array. We analyzed the changes of each spot intensity using Image J software. And then, we plotted as fold changes relative to IL-2-stimulated group (B). (C) To find transcription factors activated by IL-2 and inhibited by TGF-β1, we scored relative fold changes, and plotted in reversed bi-axis X-Y graph. The X-axis displays the fold changes of transcription factor binding activity in media-treated group relative to IL-2-stimulated group, whereas the Y-axis displays the fold changes of transcription factor binding activity in TGF-β-stimulated group in the presence of IL-2 relative to IL-2-stimulated group. (D) 12 TFs are selected and their activities are compared (red dots in C). Results are given as mean ±SD of duplicate measurements.

2. Effects of the inhibitors of selected TFs

To investigate the influence of each transcription factor on NK cell activity, we treated inhibitors of each transcription factor to NK-92 cells at low-dose ranging from 1nM to 1μM. Inhibitors of the selected TFs are shown in Figure 2A. Since rapamycin, a specific mTOR inhibitor, was well known to inhibit NK cell activity, we used rapamycin as a positive control. Because celastrol has been reported to disrupt Myb/p300 interaction and thereby inhibit c-Myb activity[21], we used celastrol as a c-Myb inhibitor. We also used SIS3 for SMAD3/4 inhibition, SP600125 for c-Jun inhibition, lisofylline for STAT-4 inhibition, C646 for CBP/p300 inhibition, PD98059 for c-Fos inhibition, HLM006474 for E2F-1 inhibition and 10058-F4 for c-Myc inhibition.

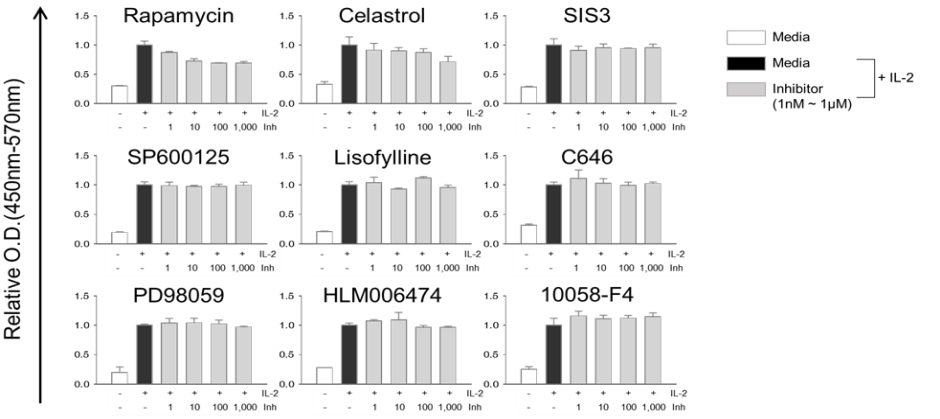
To evaluate the toxicity of each inhibitor, we measured viability of NK-92 cells at 24 hour post inhibitor treatment. We found that rapamycin inhibited cell proliferation at low-dose (Figure 2B), however, celastrol and other inhibitors didn't significantly inhibit cell proliferation at 1n M to 100 nM ranges.

To evaluate the effect of inhibitors on NK cell activity, we measured the amount of IFN-γ released. Contrary to the other inhibitor candidates, celastrol and rapamycin inhibited secretion of IFN-γ at lower dose (Figure 2C). We next investigated the effects of the inhibitors on the NK cell mediated cytotoxicity. As in the case of IFN-γ secretion, celastrol strongly inhibited NK-92 cell mediated cytotoxicity even at low concentration of 100 nM (Figure 2D). Rapamycin also inhibited the NK cytotoxicity, but other inhibitors did not. These result suggest that c-Myb, a target of celastrol, may be an important key regulator of NK cell activity.

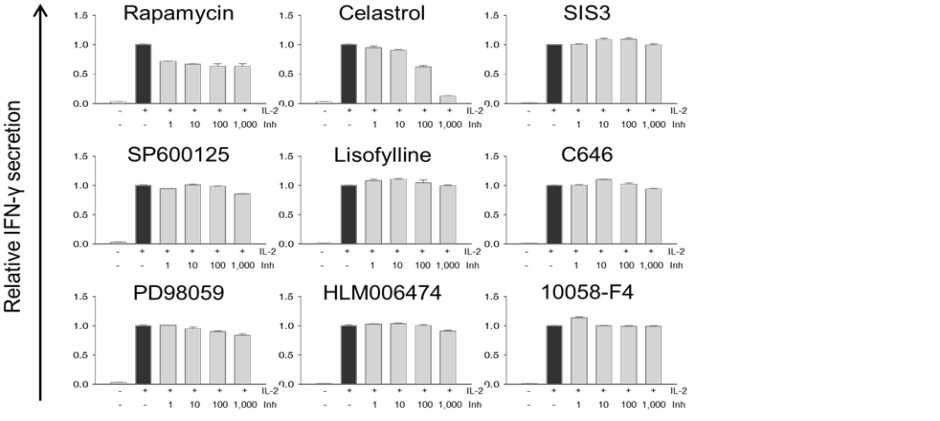
A.

#	Transcription factors /Signaling molecules	Inhibitors
1	mTOR(Positive control)	Rapamycin
2	c-Myb	Celastrol
3	SMAD-3/4	SIS3(smad3 inhibitor)
4	AP-1(c-jun)	SP600125(c-jun inhibitor)
5	Stat-4	Lisofylline
6	CBP/p300	C646
7	AP-1(c-fos)	PD98059(MEK1 inhibitor)
8	E2F-1	HLM006474
9	c-Myc	10058-F4

B.



C.



D.

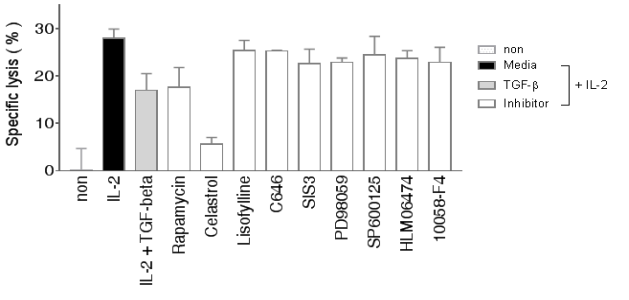


Figure 2. Effects of the selected TF inhibitors on NK-92 cell line. (A) Inhibitors of the selected TFs. To investigate the effects of the selected TFs on NK cells, we treated the inhibitors (1 nM to 1 μM) of each transcription factor to NK-92 cell lines for 24 hours (B-F). (B) Effects on the NK-92 cell proliferation. (C) Effects on the IFN-γ production by NK-92 cells. (D) Effects on the NK cell-mediated cytotoxicity, addressed by calcein-AM method.

3. Celastrol doesn't significantly affect the NK-92 cell viability at low concentration

As shown in Figure 1 and 2, c-Myb appeared to be a key regulator of NK cell activity. Before studying more the role of c-Myb in NK cells, we checked the toxicity of celastrol as a function of time and concentration. To evaluate the toxicity of celastrol on NK-92 cells, we first performed the cell proliferation assay. As shown in Figure 3A, celastrol does not significantly interrupt NK-92 cell proliferation at low concentration at 24 hour and 48 hour post treatment. However, 1μM of celastrol appeared to inhibit NK-92 cell proliferation at 48 hour post treatment. To confirm the toxicity of celastorl, we next investigated the effect of celastrol on inducing apoptosis of NK-92 cells. As shown in Figure 3B and 3C, apoptosis assay showed that celastrol did not increase the apoptosis of NK-92 cells until 100nM concentration.

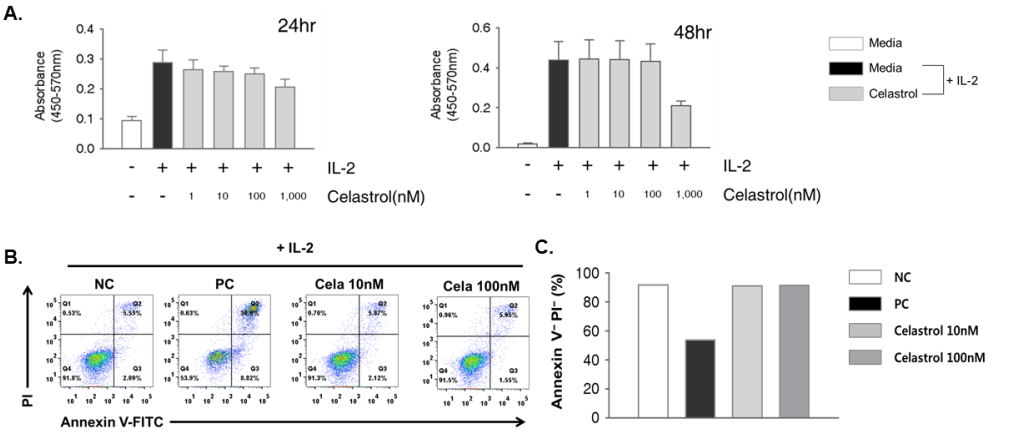


Figure 3. Effects of low-dose celastrol on the NK-92 cell proliferation and viability. (A) NK-92 cells were cultured with 10 ng/ml IL-2 with or without celastrol for indicated times. After incubation, the cells were measured for proliferation by treating CCK-8. (B and C) To assess toxicity of celastrol, we measured apoptosis level by flowcytometry. NK-92 cells were cultured with 10 ng/ml IL-2 with indicated concentration of celastrol for 24 hours. Then, the cells were immediately stained with Annexin V-FITC and PI for flowcytometric analysis (B). As a negative control (NC), NK-92 cells were stimulated with only IL-2. For a positive control (PC), NK-92 cells were stimulated with IL-2 in the presence of 5 μM of cisplatin for 24 hours. The survival rates (percentage of Annexin V^-/PI^-) of each groups were plotted in (C).

4. Celastrol diminishes NK cell activity in a dose dependent manner

We next examined the effect of c-Myb in NK cells by using a c-Myb inhibitor, celastrol. We measured the amount of IFN-γ released from NK-92 cells by IL-2 stimulation in the presence or absence of celastrol (Figure 4A). We observed that celastrol strongly reduced IFN-γ release in a dose-dependent manner (Figure 4A). NK-92 cells are well-known for their potent killing activity against various cancer cell lines. Since TGF-β inhibits NK-92 cell function, we treated 5 ng/ml of TGF-β1 as a positive control. We observed that 100 nM of celastrol inhibits NK-92 cell mediated cytotoxicity stronger than 5 ng/ml of TGF-β at any effector cell versus target cell ratio (E:T) (Figure 4B).

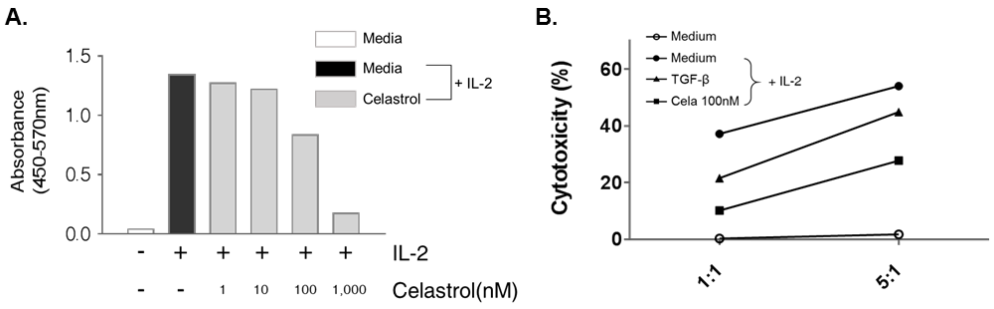


Figure 4. Low-dose celastrol inhibits NK-92 cell activity. To investigate the role of c-Myb in regulating NK-92 cell activity, we performed IFN-γ release assay (A) and NK-92 cell mediated cytotoxicity assay (B). (A) We treated 10 ng/ml of IL-2 and celastrol dose dependently. (B) Effect of c-Myb on NK-92 cell-mediated cytotoxicity. Each group of NK-92 cells were treated with 10 ng/ml of IL-2, and 5 ng/ml of TGF-β or 100 nM of celastrol for 24 hours. And then, Each groups was cocultured with calcein-AM-stained target cells (K562 cells) for 4 hours. Then calcein released from target cells was measured.

5. Myb and Myb-related signaling molecules are regulated by activation status of NK cells

We showed that DNA binding activity of c-Myb was regulated by IL-2 and TGF-β in NK-92 cells (Figure 1). Here, we demonstrate that expression of c-Myb is also regulated by activation of NK cells. c-Myb expression in NK-92 cells was detected by immunoblotting after treatment with IL-2 for 24 hours (Figure 5A). We found that Myb expression increased by IL-2 stimulation and decreased by TGF-β treatment (Figure 5A). Nemo-like kinase (NLK) is reported as a negative regulator of Myb by inducing degradation with phosphorylation.[22] We observed that NLK expression increased in IL-2 starved group and TGF-β treated group. Next we measured c-Myc expression pattern. c-Myc is known as a target gene of c-Myb.[23,24] So we hypothesized that if TGF-β inhibits c-Myb, c-Myc expression level would decrease in TGF-β treated group. As we expected, NLK expression increased and c-Myc expression level decreased after TGF-β treatment (Figure 5A).

Since c-Myb has been reported to be autoregulated,[25,26] we next inhibited c-Myb by celastrol treatment, then checked the expression level of c-Myb (Figure 5B). As we expected, celastrol dose-dependently inhibited Myb expression as much as TGF-β did.

We next investigated the expression patterns of c-Myb in human primary NK cells. To assess c-Myb expression level, human NK cells were stimulated with IL-2 and c-Myb expression was monitored by flow cytometry (Figure 5C). We found that the c-Myb expression level was upregulated at 96 hour post IL-2 stimulation in primary NK cells. These results indicate that the expression level of c-Myb is correlated with the activation status of NK cells.

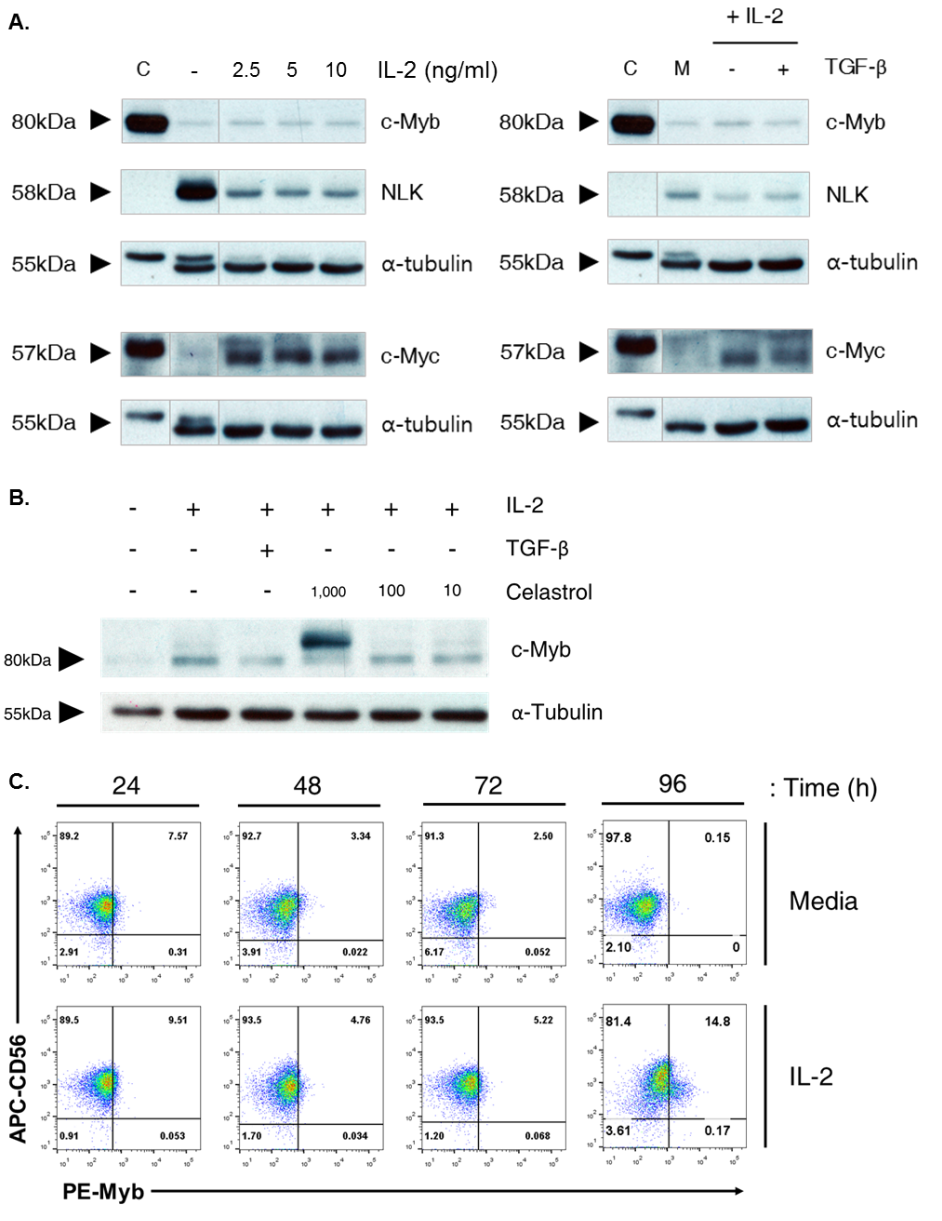


Figure 5. Expression patterns of Myb and Myb-related signaling molecules in NK-92 cells and human primary NK cells. (A and B) To investigate the Myb expression patterns in NK-92 cells, we treated IL-2 with TGF-β or celastrol for 24 hours. Then, the cells were harvested, washed and lysed in RIPA buffer. Total 30μg of protein extracted from the cells were used for immunoblot analysis. (A, left pannel) Effects of IL-2 on the expression of Myb and Myb-related signaling molecules. (A, right pannel) Effect of TGF-β on the expression of Myb. Jurkat T cells were used as a positive control (C, control) to compare Myb expression patterns with NK-92 cells. (B) Effect of celastrol on Myb expression. (C) To investigate the Myb expression patterns in human primary NK cells, 1×10^6 cells of isolated human primary NK cells were stimulated with IL-2 (5 ng/ml) or media for the indicated time, after which we monitored c-Myb expression by flow cytometry.

6. Celastrol regulates NK-92 cell activity through downregulation of NKG2D and granzyme B

To investigate how celastrol inhibits NK-92 cell mediated cytotoxicity, we measured the expression level of NK-92 activating receptors (Figure 6A) and cytolytic molecules (perforin and granzyme B) (Figure 6B) by flow cytometry. Since NK-92 cells lack most of the inhibitory receptors, we focused on the expression of activating receptors (Figure 6A). We observed that NKp30 and NKG2D expression levels increased by IL-2 stimulation. Interestingly, we observed that NKG2D expression level was significantly down-regulated by celastrol compared to IL-2 stimulated group, however, NKp30 expression level did not decrease by treatment of 100 nM celastrol treatment. (Figure 6A).

MicroRNA-150 has been reported as a negative regulator of c-Myb expression.[27,28] MicroRNA-150 is also known to regulate perforin expression in NK cells.[29] So we measured perforin and granzyme B expression level to confirm correlation between c-Myb expression level and cytolytic machinery expression level by treatment of celastrol (Figure 6B). At 24 hour post celastrol treatement, granzyme B expression level decreased, however, perforin expression level wasn't affected by celastrol (Figure 6B). Thus, we conclude that there is no significant relationship between c-Myb activation and perforin expression, but granzyme B expression appears to be affected by c-Myb activation.

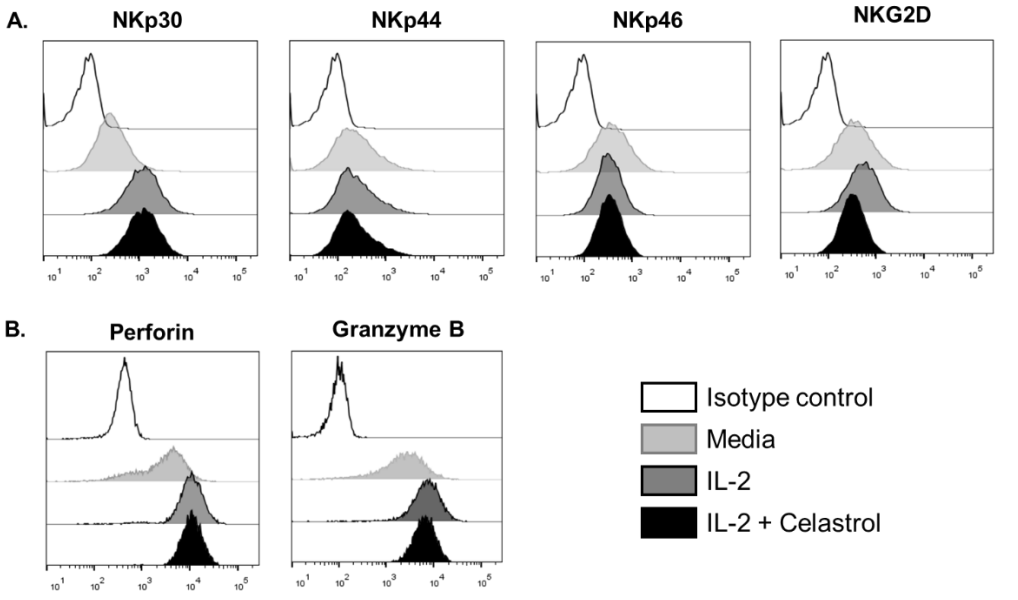


Figure 6. Effects of celastrol on the expressions of NK cell activating receptors and cytolytic molecules expression. (A-B) To identify factors affecting inhibition of NK-92-mediated cytolytic activity, we examined the expressions of NK cell activating receptors (A), and cytolytic molecules (B) by flowcytometric analysis at 24 hour after stimulation with 10 ng/ml of IL-2 in the presence or not of 100 nM celastrol.

7. c-Myb knockdown studies

To study more clearly the role of c-Myb in regulating NK cell activity, we knocked down c-Myb in NK-92 cells by using RNA interference. Knockdown of c-Myb was confirmed by real time-qPCR and Immunoblot methods (Figure 7A and B). c-Myb has been reported to influence the cell proliferation. However, knockdown of c-Myb in NK-92 cells did not make a big difference in cell proliferation between MYB shRNA group and control group (Figure 7C). At 48 hour after IL-2-stimulation, MYB shRNA-expressing NK-92 cells proliferated slightly slower than the control group (Figure 7C).

Since there was no report about correlation between transcription factor c-Myb and natural killer cell cytotoxicity, we assessed NK cell killing activity under c-Myb knockdown condition. The killing activity of MYB shRNA-expressing NK-92 cells significantly decreased compared to the control shRNA-expressing NK-92 cells (Figure 7D). This result is consistent with that obtained from celastrol treatment.

We also measured the amount of secreted IFN-γ (Figure 7E) in transduced NK-92 cells. In common with celastol treatment results, MYB shRNA-expressing NK-92 cells produced less IFN-γ than control group (Figure 7E). This result should be further investigated in their exact role in molecular mechanisms.

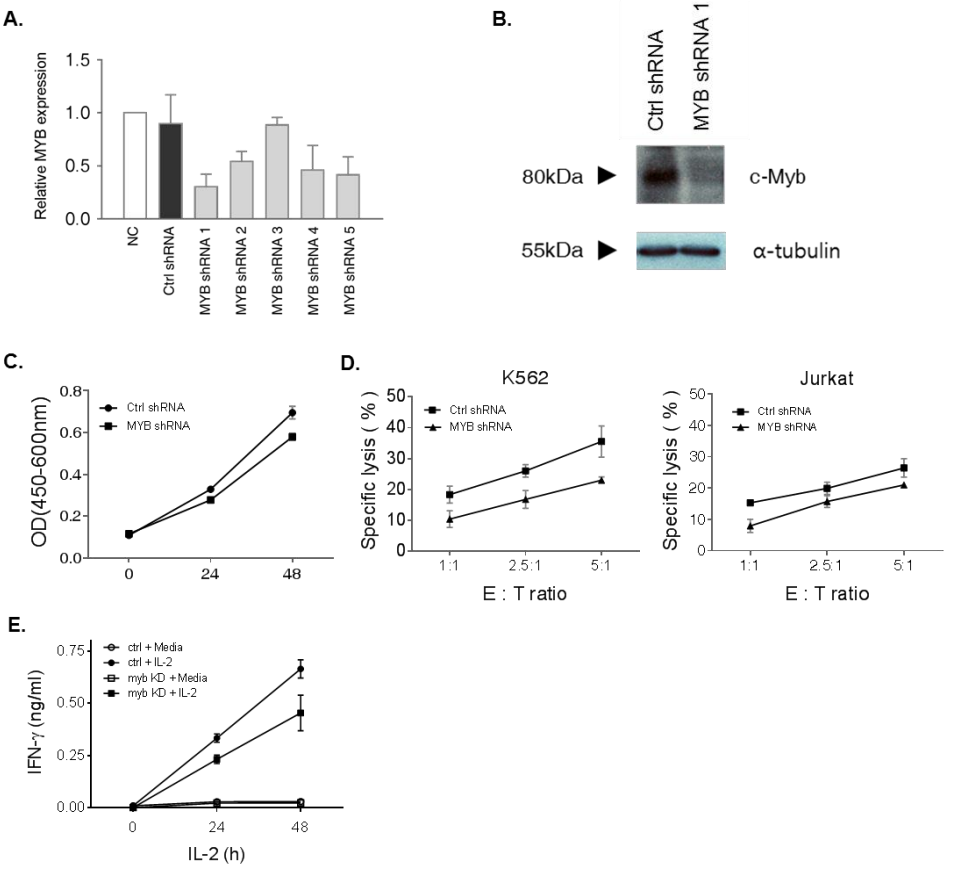


Figure 7. Effects of the knockdown of c-Myb in NK-92 cell lines. NK-92 cells were stimulated with IL-2 (10 ng/ml) for an hour, after which the cells were transduced with lentiviral expression construct encoding a control shRNA or 5 different MYB shRNA targeting different location of MYB mRNA. We evaluated the knockdown of c-Myb by performing qPCR (A) or immunoblot analysis (B). (C) For cell proliferation assay, 1×10^4 transduced cells were cultured with IL-2 (5 ng/ml) for the indicated time. At each indicated time, the amount of cells were indirectly measured by treating CCK-8 solution. (D) For cytotoxicity assay, the calcein-labeled target cells were cocultured with the transduced cells for 4 hours at indicated E:T ratio. The amount of calcein released from target cells was measured by spectrofluorometer. (E) For IFN-γ ELISA, the amount of IFN-γ released from the transduced cells was measured.

IV. DISCUSSION

Recently, c-Myb has been studied more as a regulator of NK cell development in final stage of maturation. c-Myb is known as the target of a variety of microRNAs. MicroRNA-15/16 was reported as a negative regulator of c-Myb.[30] By deletion of microRNA-15/16 in transgenic mouse, c-Myb expression level was increased resulting accumulation of immature NK cells.[30] MicroRNA-150 also targets c-Myb directly. A report shows that microRNA-150 differentially regulates the development of NK and iNKT cell lineages by targeting c-Myb.[31]

However, there were evidences that c-Myb expression is directly correlated with activation status of NK cell. c-Myb expression was upregulated by IL-2 and interferon-beta (IFN-β). And it was expected that increased natural killer cell cytotoxic activity has a correlation with increased c-Myb expression level.[32] Novel IL-2 receptors on NK cells mediated IL-2-induced activation of natural killer cells. c-Myb binding activity was increased by IL-2 treatment on NK cell lines.[33] As a well-known microRNA-150 target, Myb expression level was upregulated in mouse NK cells in microRNA-150 deleted condition. However, on the activated status of mature NK cell, microRNA-150 expression level was downregulated in mature NK cells.[29]

Although c-Myb, as one of oncogenes, is considered as a target of anti-cancer therapies, we hypothesized that c-Myb is a regulator of NK cell activities. c-Myb expression in stimulated mature NK cells already has been reported.[34] Although c-Myb expression level of mature NK cell is much lower than their progenitor cells[30], we showed that c-Myb binding activity and expression level were increased by IL-2 stimulation in human primary NK cells. Previously, our laboratory also observed that c-Myb binding activity was increased in NK-92 cells in the presence of IL-2.[20] These results provide a hint that c-myb can be one of the key factors in regulation of NK cell

activities.

In this study, we firstly report that celastrol potently inhibits NK cell activities at low molar concentration. We used celastrol, one of the quinone methides family, as a c-Myb inhibitor. Celastrol interrupts interaction between c-Myb and its coactivator p300/CBP.[21] As we expected, celastrol decreased NK-92 cell activities at low molar concentration. We also showed that celastrol also downregulated the amount of IFN-γ released from NK-92 cells without cell toxicity. Celastrol also inhibited NK-cell mediated cytolytic activity through downregulation of NKG2D and granzyme B expression. But we only observed the phenomena. Because celastrol has multiple role, such as an anti-oxidant[35], anti-inflammatory[36,37], anti-cancer[38-41], and anti-obesity[42], we expect that celastrol has many molecular targets in NK cells. We try to search more molecular targets of celastol in NK cells.

To clarify a role of c-Myb in regulation of NK cell activities, we knocked down c-Myb in NK-92 cells by using RNA interference approaches. As we expected, knockdown of c-Myb downregulated specific lytic activity of NK-92 cells up to 50% than control group with weak effect on NK-92 cell proliferation. And c-Myc, as a molecular target of c-Myb, expression was significantly downregulated by MYB gene knockdown. Because c-Myc has an important role in regulation of cell viability, we expect that c-Myc also can be an attractive target in mature NK cells. Furthermore, IFN-γ expression level was significantly reduced in c-Myb knockdowned NK-92 cells. To study exact mechanism of c-Myb in regulation of cytolytic activity and IFN-γ secretion, we are required to do more studies to describe these results.

Now, we keep in finding factors that regulate NK cell activity. As c-Myb is as one of the attractive target in regulating NK cell activities, we are looking forward to finding its specific inhibitors or activators. Because c-Myb has multiple roles in tumorigenesis, cell proliferation and differentiation, regulation of c-Myb activity in NK cell appears to

be an important goal in fine tuning of NK cell activity, considering usage in autoimmune disease therapies and cancer therapies. And here, we firstly report celastrol as a potent inhibitor of NK cell activities. As shown in this paper, celastrol potently inhibited NK cell cytolytic activity and IFN-γ secretion. We expect that celastrol could be used for anti-autoimmune therapies.

V. CONCLUSION

Here, we revealed that c-Myb is an attractive target in regulation of NK cell activities. We observed that c-Myb expression level and binding activity were upregulated in human primary NK cells and NK-92 cells under IL-2-stimulated condition. However, c-Myb expression level and binding activity were significantly downregulated by TGF-β even in the presence of IL-2. To describe these phenomena, we performed drug screening and harnessed RNA interference. As we expected, inhibition of c-Myb binding activity potently downregulated NK cell-mediated cytolytic activity and IFN-γ secretion without cell toxicity in the presence of celastrol. In accordance with pharmaceutical c-Myb inhibitor studies, we observed that Knockdown of c-Myb downregulated NK-92 cell-mediated cytolytic activity and the amount of IFN-γ secreted by NK-92 cells. In summary, these results show that c-Myb could be an attractive target in regulating NK cell activities, considering clinical usages of NK cells in anti-cancer therapies and the treatment of autoimmune diseases.

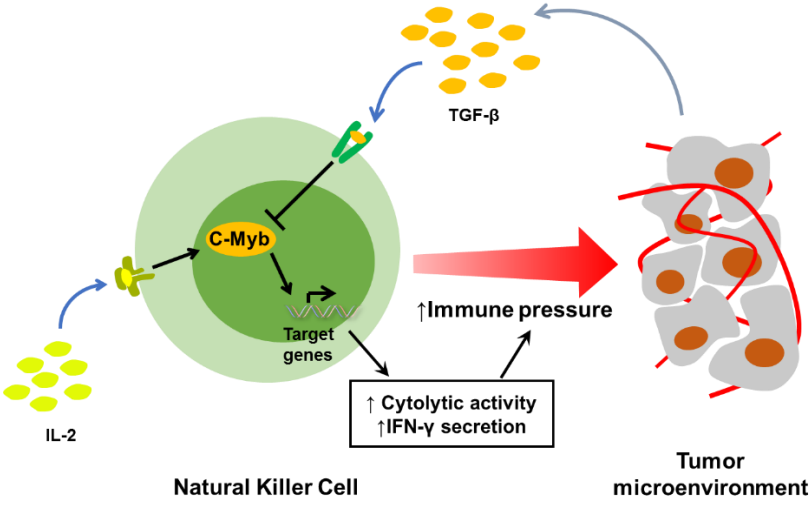


Figure 8. Graphical abstract

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ABSTRACT (IN KOREAN)

자연살해세포의 활성 조절에 대한 전사인자 c-Myb의 역할

< 지도교수 김종선 >

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신희욱

자연살해세포는 숙주의 암면역반응에서 중요한 역할을 한다. 자연살해세포의 활성도는 암 진행에 대한 중요한 기준이므로, 자연살해세포의 활성을 조절하는 요소를 탐색하는 것은 중요하다. 자연살해세포는 체내 여러가지 세포가 분비하는 사이토카인인 제 1형 인터페론 (인터페론-알파, 인터페론-베타), 인터루킨-2, 인터루킨-12, 인터루킨-15, 인터루킨-18 등에 의해 활성화된다. 한편, 암미세환경에서 자연살해세포의 활성은 여러가지 요인에 의해 감소한다. 여러가지 암미세환경에 용해되어 있는 요인들 중, 종양증식인자-베타는 활성요인인 인터루킨-2 존재 하에 자연살해세포를 가장 강력하게 저해한다고 보고되어 있다. 이 논문에서 우리는 인터루킨-2와 종양증식인자-베타 (TGF-β)에 의해 동시에 영향을 받는 전사인자를 단백질/DNA 어레이를 통해 탐색하였다. 간추려진 12가지 전사인자 후보에 대한 화학 저해제를 낮은 농도에서 처리하여 자연살해세포의 활성을 측정하였다. 12가지

전사인자 후보 중 c-Myb의 저해제로 알려진 celastrol이 자연살해세포 활성 저해효과가 가장 큰 것으로 나타났다. Celastrol은 자연살해세포주인 NK-92의 세포용해활성과, 인터페론-감마의 분비를 효과적으로 억제하였다. 또한, 유세포분석을 통해서 celastrol이 자연살해세포의 활성수용체로 알려진 NKG2D의 발현과 표적 암세포 융해에 가장 큰 역할을 하는 것으로 알려진 그랜자임 B의 발현을 저해함을 밝혔다. 더욱 심층적인 c-Myb의 역할을 확인하기 위해 c-Myb의 발현을 RNAi 기술을 이용하여 NK-92에서 감소시켰다. 그 결과, 자연살해세포의 세포용해활성과, 인터페론-감마 분비 능력을 대조군에 비해 약 50%정도 낮추었다. 결과적으로 이 논문은, 자연살해세포 내에서 c-Myb이 자연살해세포의 활성 조절에서 중요한 역할을 함을 밝힌다. 동시에 c-Myb의 활성이 조절된 자연살해세포를 자가면역질환과 암치료에 대한 도구로써 응용 가능성을 제시하고 있다.

핵심되는 말 : natural killer cells, immunosuppression, tumor microenvironment, transforming growth factor-beta, interleukin-2, c-Myb, celastrol