Comparative study of the inhibitory cytokines on human natural killer cell functions

Hae mi Lee

Department of Medical Science

The Graduate School, Yonsei University

Comparative study of the inhibitory cytokines on human natural killer cell functions

Directed by Professor Jongsun Kim

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This certifies that the Master's Thesis of
Hae mi Lee is approved.

Thesis Supervisor : Jongsun Kim

Thesis Committee Member#1: Jae Myun Lee

Thesis Committee Member#2: Hyoung-Pyo Kim

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ABSTRACT

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The Graduate School, Yonsei University

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Natural killer (NK) cells have potent killing effects and produce several cytokines in response to tumor cells. On the other hand, tumors escape from the host defense system by forming an immunesuppressive network which contains immunosuppressive cytokines. This study focused on the fact that tumor that infiltrated NK cells are inhibited, and the hypothesis that there might be conditions highly related to NK cell inhibition in tumor microenvironment.

In this study, the effect of TGF-β, IL-10, and IL-4, inhibitory cytokines hereafter, on IL-2 activated human NK cells was compared in conditions where the cytokines were put separately or together. The aim of this study is to find out the most potent inhibitory condition of NK cells. The results are as follows. Each of the inhibitory cytokines and the combinations of them inhibited IFN-γ secretion, NK cell proliferation, and the expression of NKp44, NKp46, and NKG2D. Also the inhibitory cytokines affected NK cell degranulation and finally, abrogated the target cell lysis effect of the NK cells. However, they had the little effect on NKp30, TRAIL, Fas L, NKG2A, and 2B4 expression. Among the three cytokines, TGF-β showed the most

potent inhibitory effect. Furthermore, the individual effects of TGF-β were as potent as the combined effects of TGF-β and IL-10/IL-4. Thus, either with or without other cytokines, TGF-β worked as the core cytokine in terms of inhibiting overall NK cell function.

Next, the molecular mechanism of NK cell inhibition caused by TGF-β was examined on NK-92 cell. TGF-β reduced tyrosine phosphorylation of Syk and an expression of c-myc, whereas an expression of GRB2 and the phosphorylation of ZAP70, STAT5, p38, ERK, IKBα, PTEN, AKT, and FAK were not affected by TGF-β. Then specific transcription factors affected by TGF-β were identified through profiling activities of transcription factors in NK-92 cell. It was found that the IL-2-induced c-Myb, AP-1, CREB, and AR activity were completely suppressed by TGF-β treatment. TGF-β also partially reduced the activity of STAT-5 and NFAT. And TGF-β completely suppressed CBF, ER, FAST-1, and MRE, which are constitutively activated regardless of IL-2 stimulation.

In conclusion, This study proved the significance of TGF-β on NK cell inhibition by comparative analysis and suggested biomarkers susceptible to TGF-β-mediated inhibition of NK cells. The goal of our study is to provide an insight into the effects of TGF-β on NK cell immune modulation and an effective therapeutic strategy that enhances NK cell activity.

Key words: natural killer cell inhibition, TGF-β, IL-10, IL-4, c-myc, syk, c-Myb, AP-1, CREB, STAT5

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I. INTRODUCTION

Natural killer (NK) cell is a lymphocyte of the innate immune system that is immediately able to lyse tumor cells or virus-infected cells without prior sensitization[1]. NK cells recognize target cells directly through inhibitory or activating receptors. The balance between those signals determines NK cell triggering. The major activating receptors are NKG2D, NKp30, NKp44, and NKp46 which recognize target cell ligands. Inhibitory receptors are Killer cell immunoglobulin-like receptor (KIR), CD94/NKG2A receptor family, and 2B4 which interact with MHC class I molecules on target cells. Once activated, NK cells kill tumor cells by releasing cytotoxic granules containing perforin and granzyme. Also, the apoptotic target cell death is induced by a direct interaction between tumor necrosis factor (TNF), Fas ligand, and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) expressed on NK cells. Activated NK cells secrete interferon γ (IFN-γ) and contribute to an indirectly immune response through crosstalk with dendritic cells[2].

NK cells hamper tumor growth and metastasis[2]. Several studies have indicated that

tumor tissues are infiltrated by NK cells[3], and in many cases, the infiltration of NK cells has correlation with improved prognosis of cancer patients[4]. However, clinical investigations reveal that tumor-infiltrating or peripheral blood NK cells of cancer patients display impaired functions[5,6] and impaired NK cell function is related to poor prognosis[7]. Recently, several studies have reported that tumor cells make up microenvironment that inhibits NK cells' activity and promotes survival of the tumor cells. Tumor cells employ suppressive mechanisms, including soluble factor secretions such as TGF-β, shedding NKG2D ligands like MICA and ULBP, and recruitment of suppressive immune cells, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs)[8]. Tregs inhibit NK cells by a direct interaction with membrane-bound TGF-β[9]. These factors are mainly known to cause NK cell dysfunction by causing defective expressions of activating receptor, which are NKp30, NKp44, and NKG2D, and downstream signaling molecules so that tumor cells can escape from the direct recognition of NK cells[10].

Thus, it is a crucial issue that overcoming suppressive mechanisms of NK cells which interrupt anti-tumor immune response of cancer patients. However, most of NK cell therapies aim to only improve NK activity, disregarding the suppressors derived from pathogenic circumstances of cancer. In addition, this type of therapies such as cytokine treatment, adoptive cell transplantation are cytotoxic, elaborate, intricate and cost-demanding therapy[11]. Therefore, finding a suppressive mechanism and central biomarkers highly related to in NK cell inhibition can be a promise strategy to maintaining normal NK cell function. However, suppressive mechanisms of NK cells are scarcely known.

Among suppressive factors, this study focused on three immune-regulatory cytokines, TGF-β, IL-10, and IL-4 (inhibitory cytokines hereafter). They have a higher serum level of cancer patients than that of average individuals. TGF-β is

reported to be secreted 1~20 ng/ml in multiple cancer patients[12]. TGF-β reduces NKG2D, NKp30 expression, IFN-γ secretion, T-bet expression, and killing effect of NK cells[13]. TGF-β activates transcription factor SMAD 2, 3, 4, ERK 1/2, p38, and PI3K kinase pathway that regulate cell survival, growth, and inflammation[14]. IL-10 and IL-4 are immune-regulatory cytokines produced by Th2 type cells. IL-10 is reported to be secreted 10~50 pg/ml in late stage cancer patients[12,15]. IL-10 is known to reduce IFN-γ, TNF-α secretion and NK activity in cancer cells[16]. IL-10 and TGF-β also induce Tregs[17]. IL-10 activates transcription factor STAT3. However, inhibitory mechanism is not clear at molecular level[18].

IL-4 is detected 10~70 pg/ml in a malignant tumor[19]. Although the effects of IL-4 on NK activity are controversial, several studies reported that IL-4 inhibits IFN-γ secretion, proliferation of NK cells in a sex hormone dependent cancer such as breast cancer[20]. IL-4 activates transcription factor STAT6 and Akt/PI3k which regulate cell growth[21]. Meanwhile, molecular mechanism of IL-4 that causes NK cell inhibition is unknown.

In this study, *in vitro* systemic comparative study of the inhibitory effect of TGF-β, IL-10, and IL-4 on NK cell functions was done. Aims of this study are to investigate the most vulnerable condition for NK cell effector functions, and to identify signaling intermediates that target the condition. Results demonstrated that TGF-β was the core molecule that inhibits NK cell activity in single and combination treatment. IL-2-activated NK cells were relatively more susceptible to suppression than resting NK cells. It was also identified that TGF-β reduced tyrosine-phosphorylation of Syk and total c-myc expression upregulated by IL-2 stimulation. In addition, it was found that promoter binding activity of transcription factors c-Myb, AP-1, CREB, AR, and STAT5, which is enhanced by IL-2, was suppressed upon TGF-β treatment.

II. MATERIALS AND METHODS

1. Antibodies and reagents.

The antibodies and reagents used for this study are as follows; FITC mouse anti-human CD56, PE mouse anti-human CD107a, APC mouse anti-human CD25, FITC mouse anti-human CD3, PerCP-cy5.5 mouse anti-human CD14, PE mouse anti-human CD19, PE-cy7 mouse anti-human CD16, APC mouse anti-human CD56, PE-cy7 mouse anti-NKp46, APC mouse anti-NKG2D, and FITC mouse anti-2B4 were purchased from BD Pharmingen (San Diego, CA, USA), PE mouse anti-human NKp30, PE mouse anti- human NKp44 were purchased from Beckman Coulter Company (Marseille, France), PE mouse anti-human FasL and PE mouse anti-human TRAIL were perchased from Biolegend (San Diego, CA, USA) Percp-Cy5.5 mouse anti-NKG2A was purchased from R&D systems (minneapolis, MN, USA). Rabbit anti-phospho-SMAD3, Rabbit anti-phospho-p38, Rabbit anti-phospho-STAT5, mouse anti-ZAP70, mouse anti-phospho-FAK, and mouse anti-phospho-IKBα were obtained from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-phospho ERK, mouse anti-syk, mouse anti-c-myc, and mouse anti-α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-phosphotyrosine and mouse anti-GRB2 were purchased from BD transduction laboratories (San Diego, CA, USA). Recombinant human (rh) -Interleukin 2, rh-interleukin 4, and rh-interleukin 10 were purchased from ATgen (Seongnam-Si, South Korea) and rh-human TGF-β1 was purchased from Cell Signaling Technology (Danvers, MA, USA).

2. Cell lines and cell culture.

The human NK cell line; NK-92 cell; was maintained in Minimum Essential Medium α (MEM α) (Gibco, New York, NY, USA) supplemented with 12.5%

heat-inactivated FBS, 12.5% heat-inactivated horse serum (Gibco), 0.2 mM Myo-inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, penicillin, streptomycin and 5 ng/ml of IL-2, and incubated at 37˚C with 5% CO^2. The cells were maintained with the density of $1\sim2\times10^5$/ml and reseeded every 2 or 3 days. The cells were washed with PBS before they remove membrane bound IL-2. The chronic myeloid leukemia cell line K562 was maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics, and incubated at 37˚C with 5% CO^2. Human NK cells were activated with indicated combinations of 2 ng/ml of IL-2, 10 pg/ml of TGF-β, 10 pg/ml of IL-10, and 10 pg/ml of IL-4 for 48 hours. NK-92 cell lines were activated with indicated combination of 2 ng/ml of IL-2, 1 ng/ml of TGF-β, 1 ng/ml of IL-10, and 1 ng/ml of IL-4 for 48 hours.

3. Isolation of NK cells.

Natural killer cells were isolated from the whole blood of healthy donors by a negative selection using a Rosettesep^{TM} NK enrichment antibody cocktail (StemCell Technologies, Vancouver, B.C., Canada). The antibody cocktail contained anti-CD3, anti-CD4, anti-CD19, anti-CD36, and anti CD-66b antibodies. 50 microliters of the antibody cocktail per 1ml of whole blood was mixed and incubated for 20 minutes at room temperature. Then the sample was diluted with the same volume of PBS containing 2% FBS. The diluted blood with PBS was placed onto a Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifugated for 20 minutes at 1200 x g at room temperature. The NK cell was collected and washed twice with PBS containing 2% FBS, then re-suspended RPMI medium containing 10% FBS and used for the following experiments. Cells were >91% $CD56^+$, as evaluated by FACS analysis.

4. Sandwich Enzyme-linked immunosorbent assay (ELISA).

The level of secreted IFN-γ and TNF-α were measured by commercial IFN-γ ELISA kits (ATgen, Seongnam-Si, South Korea) and TNF-α ELISA sets (BD Biosciences San Diego, CA, USA) followed the original instructions. Whole blood samples (1 ml/sample) were incubated for 24 hours after cytokine treatments and human NK cells (5 x 10^5 /sample) and NK-92 cells (1 x 10^5 /sample) were incubated for 48 hours after treatment of each cytokine. Plasma or culture supernatants were harvested and separated by centrifugation at 12000 rpm for 5 minutes. Collected samples were treated immediately or within 48 hours after being frozen. 100 microliters of serial dilutions of standard, control or sample were carried in duplicate on 96-well microplates precoated with the appropriate antibodies and incubate for 2 hours at room temperature. Each well was washed 4 times with wash buffer and 100 microliters of HRP conjugated secondary antibodies was added. After 1 hour incubation at room temperature, wells were washed 8 times and 100 microliters of substrate solution (TMB) was added to each well, incubated for 30 minutes at room temperature and protected from light. The optical density of each well was observed by a microplate reader set to 450nm right after being added 100 microliters of stop solution..

5. CCK-8 cell proliferation assay.

CCK-8 assay was used for NK cell proliferation. Either 100 microliters of NK cell (1 x 10^5/well) or NK-92 cell suspension (5 x 10^5 /well) was dispensed in triplicate in a 96 well round-bottom plate. The plate was incubated for 44 hours at 37˚C with 5% CO^2. 10 microliters of CCK-8 solution (water-soluble tetrazolium salt) was added to each well of the plate and incubated for 4 hours. The absorbance was measured at 450 nm using a microplate reader.

6. Multicolor flow cytometry.

Flow cytometry assay was used for analysis of various cell surface molecules expressed on NK cells. Cells (5 x 10^5 /sample) were washed twice with PBS containing 2% FBS and stained with fluorochrome-conjugated antibodies for 30 minutes on ice the dark. Then, the cells were washed twice with PBS containing 2% FBS. The fluorescence was measured using BD LSR II. Alternatively, during primary NK cell experiments, compensation beads (mouse I, kappa) (BD Biosciences, San Diego, CA, USA) were used for the multicolor compensation.

7. ^{51}Chromium release assay.

The ^{51}Cr release assay was used to measure cytotoxic activity of NK cells against K562 cells. 50 μCi of ^{51}Cr (NEN, Boston, MA, USA) was added per 5 ×10^5 K562 cells and incubated for 2 hours at 37˚C with 5% CO^2. ^{51}Cr-labeled K562 cells were washed with PBS three times. K562 cells were plated (5×10^3 cells per 100 microliter) into a round-bottom 96 well plate. NK cells then were added to different E/T ratios in triplicate in each well and co cultured for 4 hours. Supernatants were collected into gamma counter tubes (PerkinElmer, Boston, MA, USA), and a radioactivity was measured by gamma counter (PerkinElmer). The specific lysis was calculated by the formula; Percentage of specific cytotoxicity = (experimental cpm- spontaneous cpm) / (maximum cpm- spontaneous cpm) x 100. Maxium ^{51}Cr release was reliant onsupernatants of lysed target cells incubated with 2% Triton X-100. Spontaneous release was dependent on K562 cells incubated in medium without NK cells.

8. CD107a degranulation assay.

CD107a assay was used to measure the degranulation of NK cells in response to stimulation with indicated cytokines. After 48hour culture, 1×10^4 NK cells or 5×10^3

NK-92 cells in 100 microliter assay medium were dispensed in round-bottom 96 well plate. Then 4 microliter PE mouse anti-CD107a was added to the culture media and the cells were incubated with the same number of K562 target cells in 100 microliter medium for 4 hours at 37˚C in 5% CO^2 incubator. Control samples were incubated without target cells to detect spontaneous degranulation. Thereafter, the samples were stained with FITC mouse anti-CD56 on ice for 30 minutes and then flow cytometric analysis was conducted with these samples.

9. Immunoprecipitation and Western blot.

$1\sim5\times10^6$ NK-92 cells were treated with cytokines or PBS for 5 minutes or 30 minutes at 37˚C. After stimulation, the cells were pelleted and washed 3 times with ice-cold PBS. The cells (10^8 cells/ml) were lysed in RIPA buffer (0.15 M NaCl, 1% Nonie P-40, 0.1% SDS, 50 mM Tris (pH 8.0)), supplemented with protease, phosphates inhibitor cocktail and 0.5 M EDTA (Thermo scientific, MA, USA). Protein concentration was determined by BCA protein assay (Thermo scientific).

For immune-precipitation, 500 μg of cell lysate were pre-coated with 1 μg of Syk-Ig and incubated at 4˚C overnight. 20 microliter of protein A/G plus agarose (Santa Cruz) were added to the cell lysate and incubated at 4˚C for 4 hours. After washing with lysis buffer 3 times, the samples were pelleted and the supernatants were analyzed by SDS-PAGE and western blotting. For western blot, each sample was added to sampling buffer, boiled at 100˚C for 5 minutes. And they were loaded onto 10-12% SDS-PAGE gel and transferred to methanol-activated PVDF membrane. After being blocked by a nonspecific reactivity, the membrane was probed with specific antibodies diluted in TBS-T (20 mM Tris-HCl (pH 8.0), 1.37 M NaCl, and 0.05% Tween-20) containing 5% BSA or Skim milk. Immunoreactivity was detected by using an ECL kit (Thermo scientific).

10. Protein/DNA array for promoter binding activity analysis.

Nuclear extraction was conducted prior to Protein/ DNA array. Nuclear extracts were prepared with the Nuclear Extraction kit (Panomics, Fremont, CA, USA) followed the original manual. Protein/DNA array was performed to profile activities of multiple transcription factors. Each array assay was carried out following the procedure in the Protein/DNA array kit (Panomics) user's manual. 5 μg of nuclear extract was mixed with DNA probe (10 ng/μl), and the mixture was incubated at 15˚C for 30 minutes. The reaction was loaded on the membrane of the column and incubated for 30 minutes on ice. After incubation, the columns were washed with washing buffer four times followed by centrifugation at 7000 x g and 4˚C. DNA probes were eluted with elution buffer and denatured at 95˚C for 3 minutes before being hybridized to the array membrane at 42˚C overnight. The membrane was washed twice in washing buffer I at 42˚C for 20 minutes and then twice in washing buffer II at 42˚C for 20 minutes. Membrane detection was conducted following the procedure in the manual. Enhanced chemiluminescence reagents and exposed to photographic film.

III. RESULTS

1. **Comparison of the effects of the inhibitory cytokines on IFN-γ and TNF-α secretion in whole blood.**

Since NK cells are known to predominantly produce IFN-γ and also produce TNF-α[2], the effect of TGF-β, IL-10, and IL-4 on IFN-γ and TNF-α secretion in whole blood was compared. A low concentration of cytokines which have minimal inhibitory effects, based on the serum level of cancer patients[15,19,22], were treated. The cytokines were treated with 8 ng/ml of IL-2 concomitantly since IFN-γ was not detected in resting conditions (Figure 1). As shown in figure 1A, TGF-β, IL-10, and IL-4 respectively inhibited IFN-γ and TNF-α secretion in a dose dependent way. TGF-β potently inhibited 88% of IL-2-induced IFN-γ secretion at 10 pg/ml, while it inhibited 46% of IL-2-induced TNF-α secretion at the same concentration. IL-10 reduced 54% of IFN-γ level and 57% of TNF-α level, respectively, at the maximal dose (500 pg/ml). IL-4 showed the lowest effect. IL-4 suppressed 43% and 27% of IFN-γ and TNF-α secretion at the 500 pg/ml. Then, the individual effects with the combination effects of the inhibitory cytokines were compared. In this experiment, TGF-β was treated with a lower dose (4 pg/ml) determined by low dose gradient measurement. However, combination effects did not surpass the inhibitory effect of TGF-β alone on the secretion of both IFN-γ and TNF-α (Figure 1B).

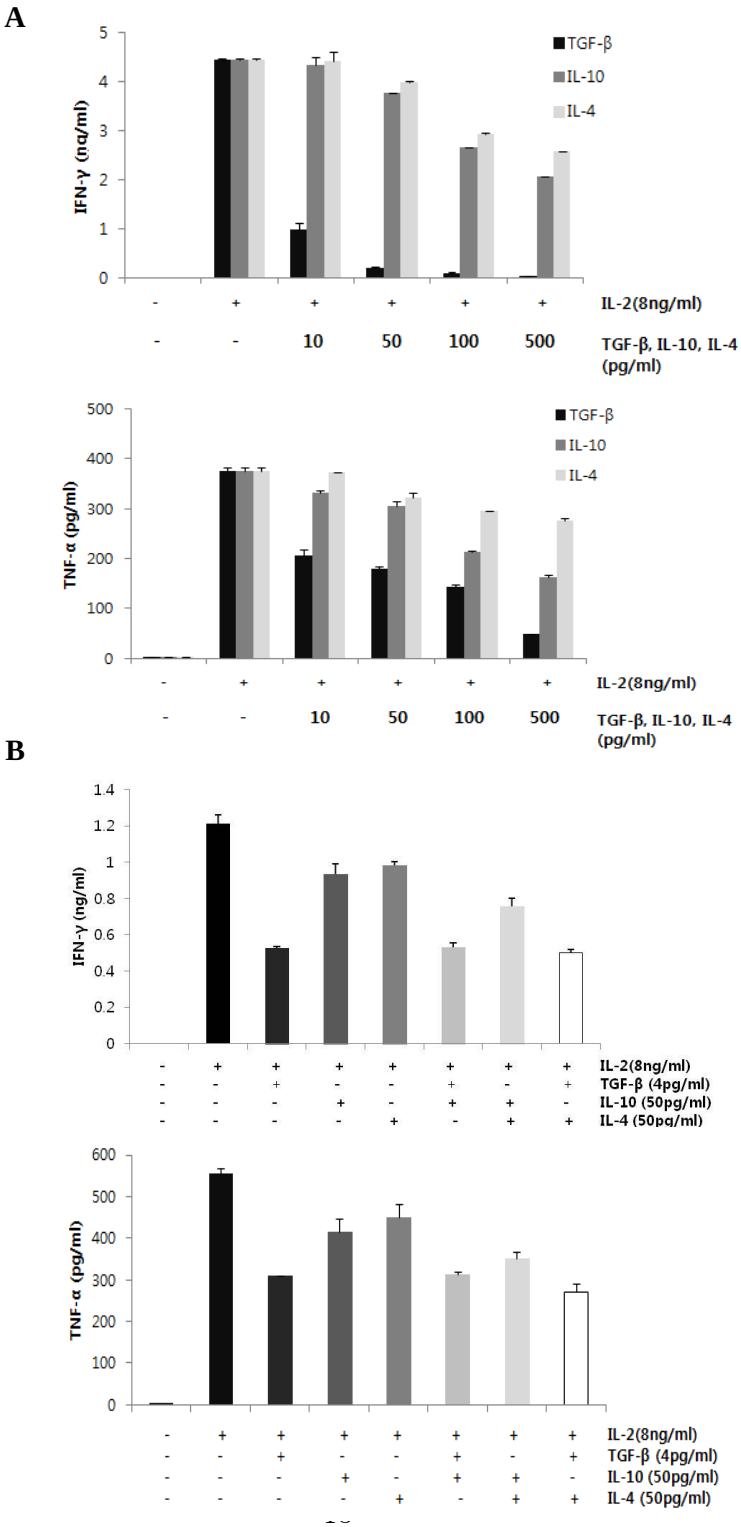

A
IFN-γ (ng/ml)
TGF-β
IL-10
IL-4
IL-2(8ng/ml)
TGF-β, IL-10, IL-4 (pg/ml)
TNF-α (pg/ml)
B
IFN-γ (ng/ml)
TNF-α (pg/ml)
IL-2(8ng/ml)
TGF-β (4pg/ml)
IL-10 (50pg/ml)
IL-4 (50pg/ml)

Figure 1. Comparison of the effects of the inhibitory cytokines on IFN-γ and TNF-α secretion in whole blood

Whole blood was cultured with the indicated cytokines for 24 hours and the supernatants were measured for IFN-γ and TNF-α secretion by ELISA. (A) Whole blood samples were treated with each cytokines in a dose-dependent manner (10 pg/ml - 500 pg/ml) in the presence of 8 ng/ml of IL-2. (B) shows the result of combined treatment of indicated cytokines. Data pooled from three independent experiments are presented.

2. Isolation of human NK cells

The peripheral blood was obtained from healthy donors. NK cells were isolated by a negative selection using RosetteSep™ antibody cocktail. Since the antibody cocktail contains antibodies that are bound to human hematopoietic cells, T cells, B cells and monocytes, they were removed by a negative selection. In Figure 2, we confirmed the purity of the isolated NK cells with T cell (CD3), B cell (CD19), monocyte (CD14), and NK cell (CD16 and CD56) markers. The average purity of isolated NK cells was 91%. Purified NK cells were used for the following experiments.

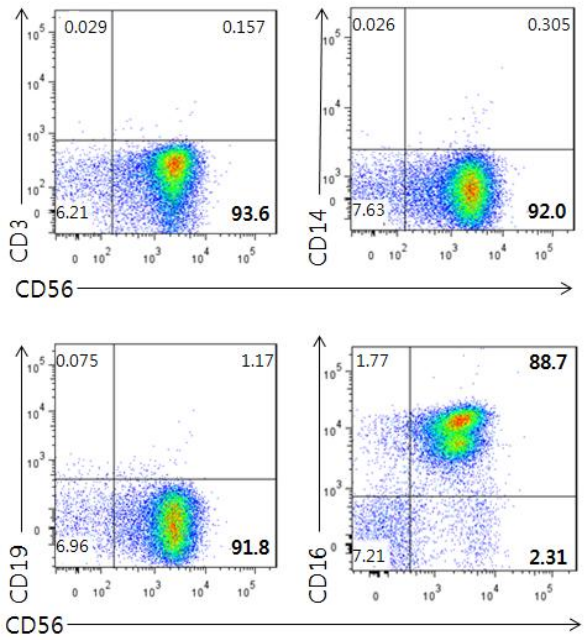


Figure 2. Isolation of human NK cells

The purity of isolated NK cells was analyzed by Flow cytometry. Isolated NK cells were stained with FITC mouse anti-CD3, Percp-cy5.5 mouse anti-CD14, PE mouse anti-CD19, PE-Cy7 mouse anti-CD16 and APC mouse anti-CD56. The average purity of NK cells was 91%.

3. Comparison of the effects of the inhibitory cytokines on IFN-γ secretion in NK cells.

After testing individual and combination effect of the inhibitory cytokines in whole blood, the same effects but on IFN-γ and TNF-α secretion were measured in NK cells. Since the serum level of IL-2 is low[23], we administered the minimum effective dose of IL-2 (2 ng/ml) to NK cells. After 48 hour stimulation, all of the inhibitory cytokines reduced IFN-γ secretion in a dose-dependent way (Figure 3A). TGF-β had the strongest effect and IL-4 showed the lowest effect similar to the whole blood experiment. However, the inhibition effect of TGF-β on NK cells was less than that on whole blood. TNF- α level was so low that could not be detected in this experiment (Data not shown). Among the combination stimulations, TGF-β and IL-4 combination induced the lowest IFN-γ concentration (Figure 3B). However, compared to TGF-β individual effect, significant inhibition was not observed in the combination treatment.

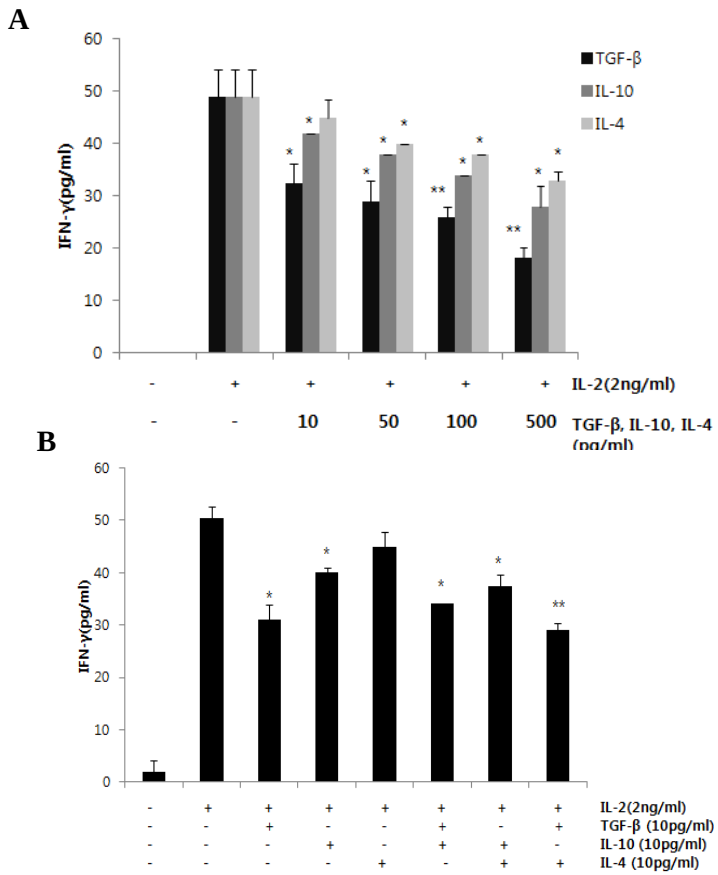


Figure 3. Comparison of the effects of the inhibitory cytokines on IFN-γ secretion in human NK cells.

Purified NK cells were incubated with indicated amount of cytokines for 48 hours. The cell culture supernatants were assayed for IFN- γ by ELISA. (A) Purified NK cells were treated with each cytokines in a dose-dependent manner (10 pg/ml - 500 pg/ml) and 2 ng/ml of IL-2. (B) shows results of combined treatment (2 ng/ml of IL-2 and 10 pg/ml of TGF-β, IL-10, and IL-4). Results are given as mean ±SD of duplicate measurements. Data pooled from three independent experiments are presented. Asterisks indicate statistical significance by independent t-test. *$p<0.05$ and **$p<0.01$

4. Comparison of the effects of the inhibitory cytokines on NK cell proliferation.

Next, the effects of the inhibitory cytokines on NK cell proliferation were compared. Each cytokine was treated with the same minimum dose (10 pg/ml) to efficiently detect the combination effects. After 48 hours of stimulation, TGF-β treatment appeared to counteracted IL-2 effects, which was a similar level to the non-treated control group. Effects of the combinations of IL-10 + IL-4 and TGF-β + IL-4 showed an addictive effect, compared to individual effects. Though the combination of TGF-β and IL-4 displayed the strongest suppressive effect, the effect was not significantly different from TGF-β single effect.

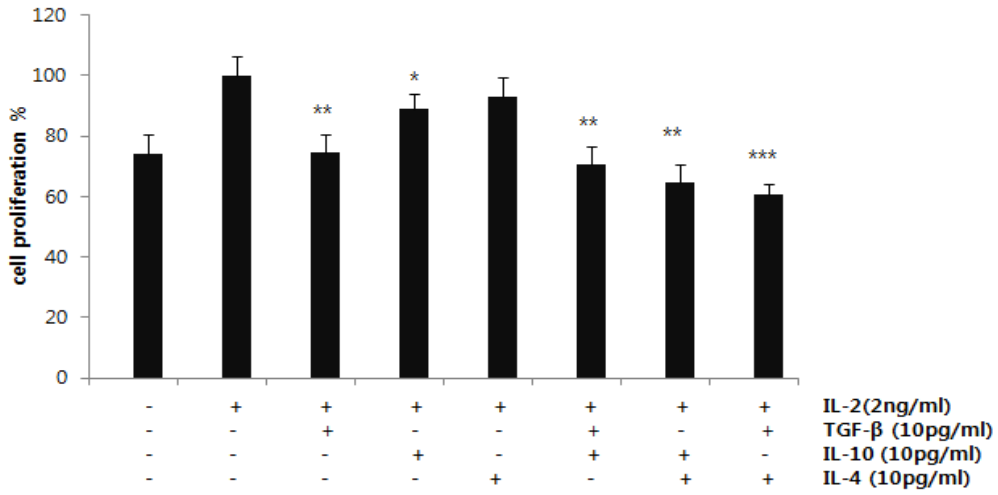


Figure 4. Comparison of the effects of the inhibitory cytokines on NK cell proliferation.

Purified NK cells were incubated with indicated cytokines for 48 hours (2 ng/ml of IL-2 and 10 pg/ml of TGF-β, IL-10, and IL-4). The cultures were pulsed with WST-8 reagents for 4 hours before assessed. Results are given as mean ±SD of triplicate measurements. Data pooled from three independent experiments are presented. Asterisks indicate statistical significance by independent t- test. *$p<0.05$, **$p<0.01$, and ***$p<0.001$

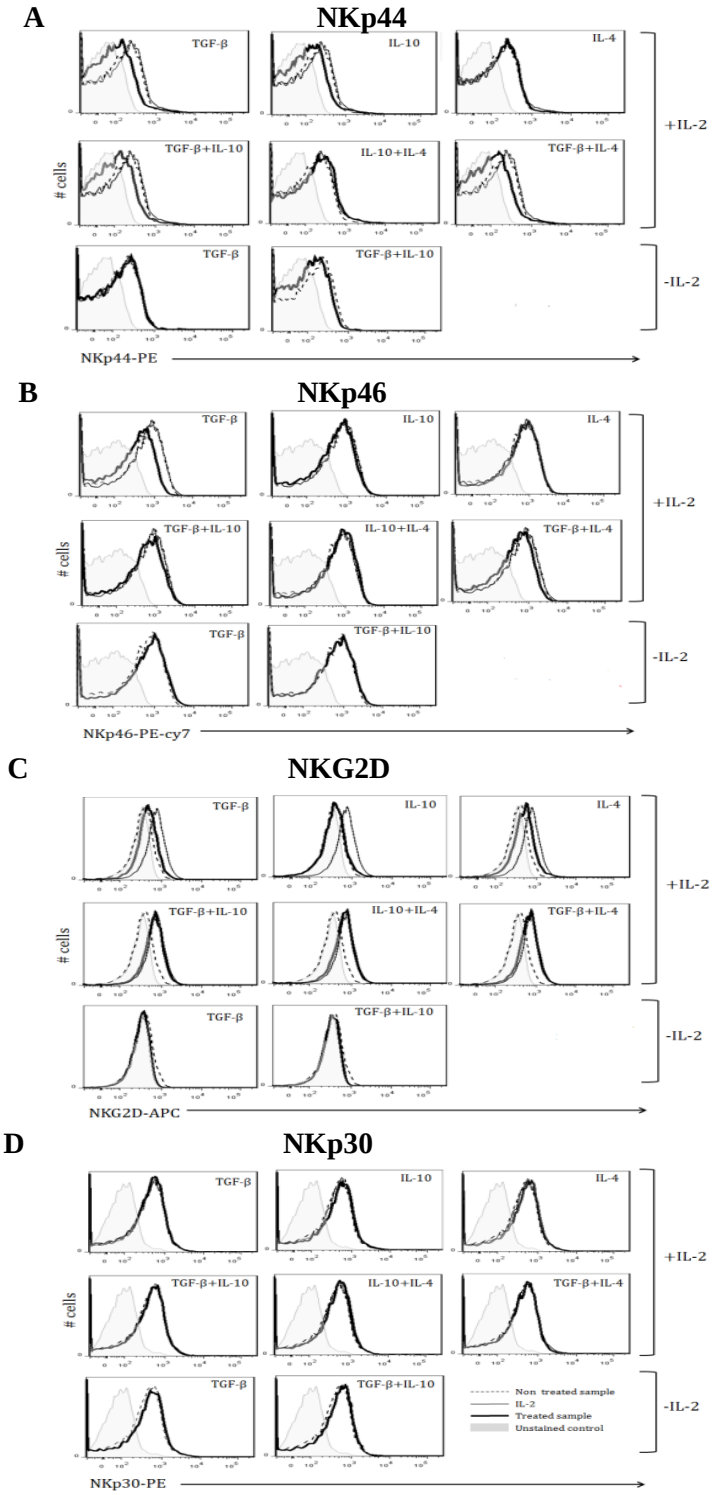
5. Comparison of the effects of the inhibitory cytokines on the expression of receptors/ligands in NK cells.

NK cells need to recognize target cell ligands by expressing activating receptors before fully activated[1]. Therefore, activating receptor expression after stimulation was analyzed in order to identify whether the cytokines affect activation status of NK cells. Purified NK cells were cultured for 48 hours and analyzed for the expression of various markers; NKp44, NKp30, NKp46, and NKG2D (Figure 5). Although IL-2 slightly upregulated NKp44, TGF-β and IL-10 down regulated NKp44 in the presence of IL-2, whereas IL-4 treated sample showed not much difference. Combinations of TGF-β+IL-10 and TGF-β+IL-4 also inhibited NKp44 expression, but the expression level of IL-10+IL-4 combination was similar to the level of IL-2 individual stimulation (Figure 5A). These findings indicated that TGF-β is critical on the down regulation of NKp44 in single and combination stimulation. Also, NKp44 expression was unaffected by TGF-β individual effect. In IL-2 exposed NK cells, only TGF-β appeared to reduce NKp46 level (Figure 5B). Like NKp44 expression analysis, TGF-β did not involve in NKp46 expression without IL-2 stimulation. Other cytokines, including the cytokine combinations, had no significant difference. Compared with unstained control and untreated staining control, NKG2D was not constitutively expressed on NK cells. NKG2D expression was upregulated by IL-2 stimulation unlike NKp44, NKp46, and NKp30. IL-10 showed the strongest effect on NKG2D down regulation. TGF-β and IL-4 also reduced the NKG2D expression level (Figure 5C). However, there was no major difference between the samples treated by the combinations of TGF-β+IL-10, IL-10+IL-4 and TGF-β+IL-4. NKG2D level was also unchanged in the resting conditions. NKp30 expression level showed an insignificant response by all cytokines including IL-2 (Figure 5D). These findings showed that TGF- β was the

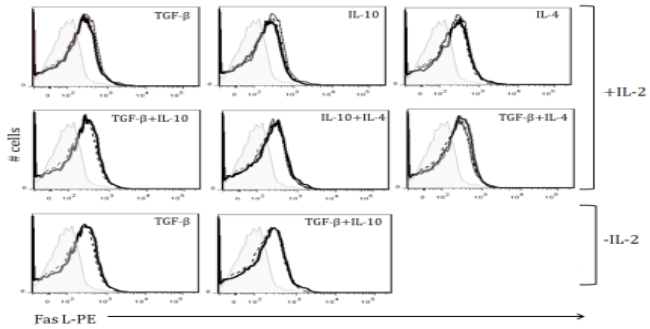
most significant cytokine to suppress activating receptor expression in IL-2 activated NK cells.

When recognizing target cells, NK cells over express death ligands. Death ligands bind to target cell receptors. The death signal is transduced and causes target cell apoptosis. The expression level of FasL and TRAIL, which are major death ligands of NK cells, was checked after the cytokine stimulation[2]. However, none of the different cytokine stimulations significantly affected expression level of FasL (Figure 5E) and TRAIL (Figure 5F).

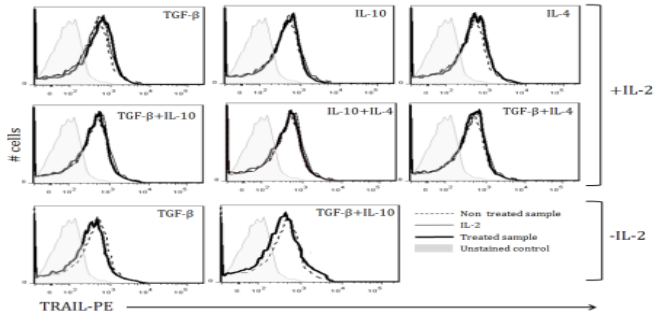
The expressions of activating receptors and inhibitory receptors are tightly regulated in NK cells. However, the effects of inhibitory cytokines on inhibitory receptor expression were not much reported. We measured expression levels of NKG2A and 2B4 after the cytokine stimulation[2]. However, none of the different cytokine stimulations were able to change expression of NKG2A (Figure 5G). IL-2, TGF-β, and TGF-β+IL-10 stimulation slightly enhanced 2B4 expression level, while other inhibitory cytokines didn't affect the expression level of 2B4 (Figure 5H).


A
NKp44
TGF-β
IL-10
IL-4
TGF-β+IL-10
IL-10+IL-4
TGF-β+IL-4
+IL-2
-IL-2
cells
NKp44-PE
B
NKp46
NKp46-PE-cy7
C
NKG2D
NKG2D-APC
D
NKp30
NKp30-PE
Non treated sample
IL-2
Treated sample
Unstained control

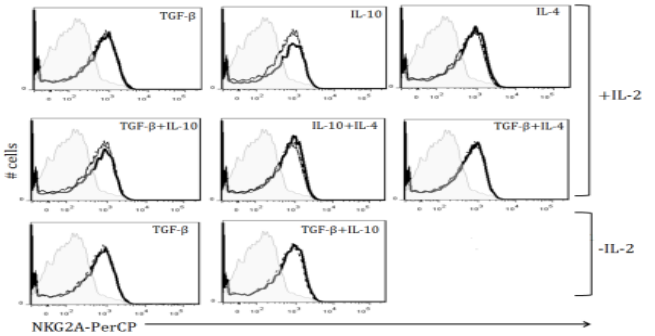
E **FasL**



F **TRAIL**



G **NKG2A**



H **2B4**

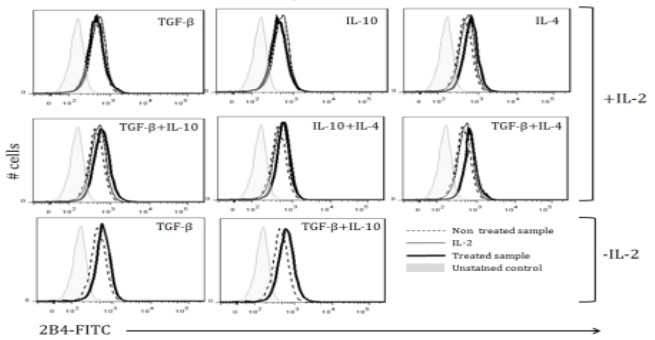


Figure 5. Comparison of the effects of the inhibitory cytokines on expression of receptors/ligands in NK cells.

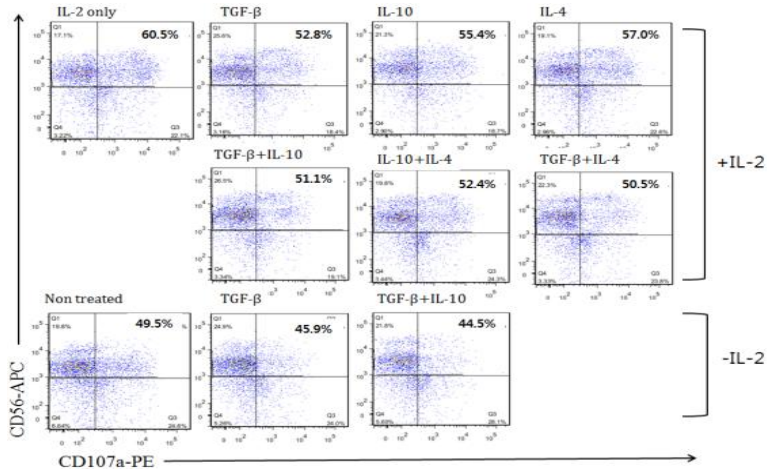
Freshly isolated NK cells were cultured with indicated cytokines for 48 hours (2ng/ml of IL-2 and 10 pg/ml of TGF-β, IL-10, and IL-4), then analyzed by flow cytometry. (Shaded area, unstained control group; dotted line, non treated group; solid line, IL-2 treated group; bold line, each inhibitory cytokine treated group (with/without IL-2)). (A) shows expression of NKp46, (B) shows expression of NKp44, (C) shows expression of NKG2D, and (D) shows expression of NKp30. (E) shows expression of Fas ligand and (F) shows expression of TRAIL. (G) shows expression of NKG2A and (H) shows expression of 2B4. Data pooled from three independent experiments are presented.

6. Comparison of the effects of the inhibitory cytokines on NK cell cytotoxicity.

One of the NK cell killing mechanisms is releasing cytotoxic granules, containing perforin and granzyme. As CD107a molecules are expressed on cell surface when perforin and granzyme are released, externalization of CD107a determines ability of NK cells degranulation[24]. K562 cells were chosen as target cells as they are susceptible to NK cell mediated killing. They do not express MHC class I molecules[25]. After 48 hour-culture in the presence of different cytokine combination, all the samples were co-incubated with K562 cells for 4 hours, then the CD107a level was analyzed (Figure 6A, B). IL-2 activated NK cells induced degranulation in response to co-culture with K562 cells. TGF-β and IL-10 each suppressed the expression of CD107a in IL-2-activated NK cells, whereas IL-4 barely affected to degranulation in NK cells. Also, the combinations of TGF-β+IL-10, IL-10+IL-4, and TGF-β+IL-4 resulted addictive effects. Moreover, TGF-β and TGF-β+IL-10 even reduced CD107a level in resting NK cells.

The effects of the inhibitory cytokines on tumor cell killing effect of NK cells were also evaluated (Figure 6C-F)**.** Data showed that at 10 : 1 ratio, TGF-β alone strongly inhibited IL-2-activated NK cytotoxicity to basal level (80%→59%). IL-10 also reduced 10% lysis effect compared to IL-2 stimulation. However, IL-4 did not affect NK cytotoxicity. TGF-β barely affected resting NK cell cytotoxicity (Figure 6C, D). Among the combinations of cytokines, TGF-β+IL-10 represented the strongest suppressive effect. However, without IL-2 stimulation, TGF-β+IL-10 barely affect NK cytotoxicity (Figure 6E, F). None of the combinations of the inhibitory cytokines exceeded TGF- β single effect. This data demonstrates that TGF-β is the core cytokine to inhibit NK cell activity and the effects of TGF- β are well observed in IL-2 activation status comparing to resting status.

A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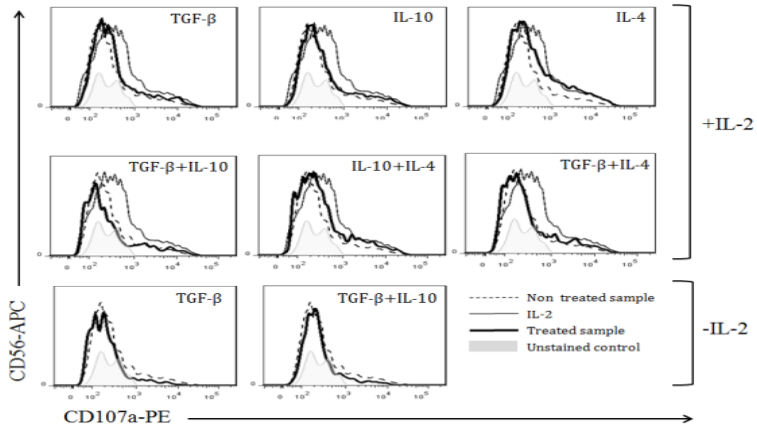
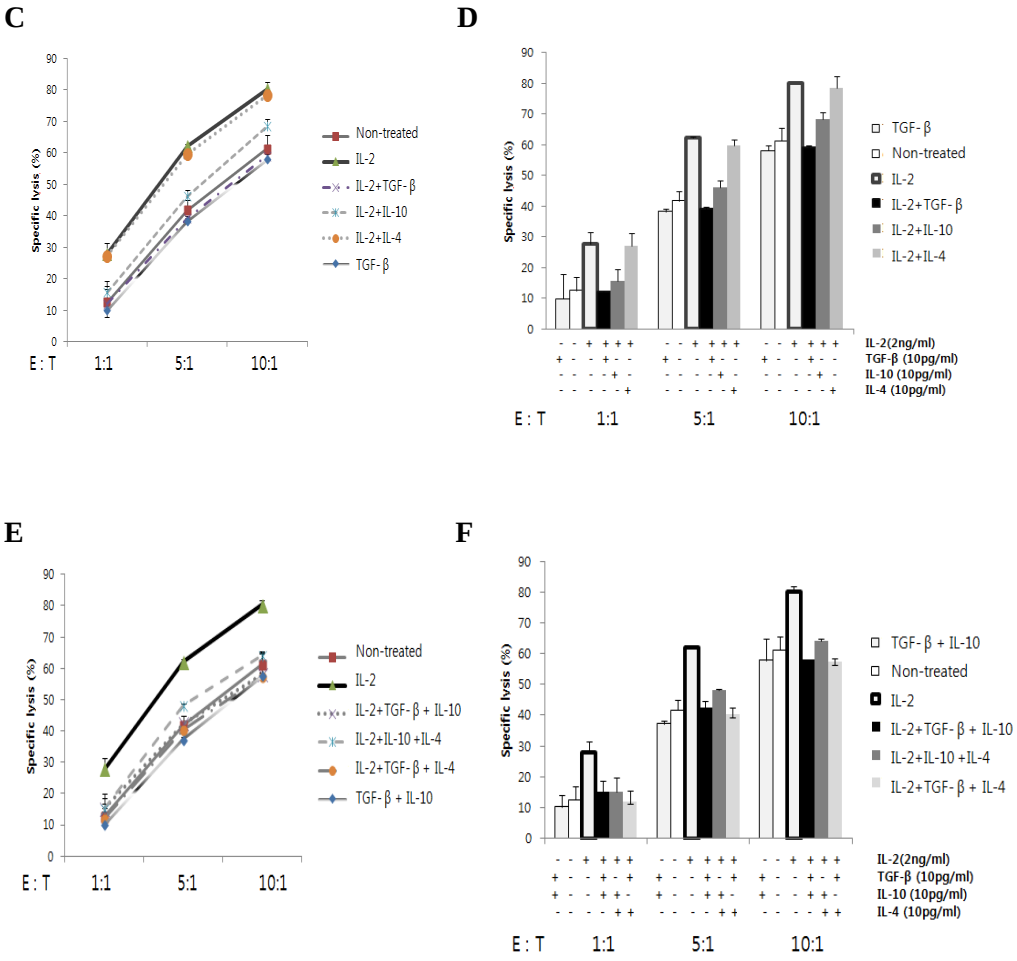
60.5%
TGF-β
52.8%
IL-10
55.4%
IL-4
57.0%
TGF-β+IL-10
51.1%
IL-10+IL-4
52.4%
TGF-β+IL-4
50.5%
+IL-2
Non treated
49.5%
TGF-β
45.9%
TGF-β+IL-10
44.5%
-IL-2
CD56-APC
CD107a-PE

B


TGF-β
IL-10
IL-4
+IL-2
TGF-β+IL-10
IL-10+IL-4
TGF-β+IL-4
TGF-β
TGF-β+IL-10
Non treated sample
IL-2
Treated sample
Unstained control
-IL-2
CD56-APC
CD107a-PE


C
Specific lysis (%)
E : T
1:1
5:1
10:1
Non-treated
IL-2
IL-2+TGF-β
IL-2+IL-10
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TGF-β
D
Specific lysis (%)
E : T
1:1
5:1
10:1
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IL-2+TGF-β
IL-2+IL-10
IL-2+IL-4
IL-2(2ng/ml)
TGF-β (10pg/ml)
IL-10 (10pg/ml)
IL-4 (10pg/ml)
E
Specific lysis (%)
E : T
1:1
5:1
10:1
Non-treated
IL-2
IL-2+TGF-β + IL-10
IL-2+IL-10 +IL-4
IL-2+TGF-β + IL-4
TGF-β + IL-10
F
Specific lysis (%)
E : T
1:1
5:1
10:1
TGF-β + IL-10
Non-treated
IL-2
IL-2+TGF-β + IL-10
IL-2+IL-10 +IL-4
IL-2+TGF-β + IL-4
IL-2(2ng/ml)
TGF-β (10pg/ml)
IL-10 (10pg/ml)
IL-4 (10pg/ml)

Figure 6. Comparison of the effects of the inhibitory cytokines on NK cell cytotoxicity.

(A-B) Freshly isolated NK cells were cultured with indicated cytokines for 48 hours in the medium alone or in the presence of the different combinations of cytokines; 2 ng/ml of IL-2, 10 pg/ml of TGF-β, IL-10, and IL-4. $5x10^4$ cells were incubated with $5x10^4$ K-562 cells and PE mouse anti-CD107a. After 4hours, the cells were stained by FITC mouse anti-CD56 then CD107a surface expression of NK cells were measured by flow cytometry. (Shaded area, unstained control group; dotted line, non treated group; solid line, IL-2 treated group; bold line, each inhibitory cytokine treated group (with/without IL-2)). (A) shows dot-plot of flow cytometry data and (B) shows the same data represented by histograms. Similar results were observed in four independent experiments**.** (C-F) Freshly isolated NK cells were cultured with indicated cytokines for 48 hours in medium alone or in the presence of different combinations of cytokines, harvested, plated at different E:T ratios with Cr-labeled K-562 cells and a 4-h cytotoxicity assay was performed. (C-D) NK cells were treated with single inhibitory cytokines. Single treatment data are displayed on line graph (C) and bar graph (D). (E-F) NK cells were treated with different combination of inhibitory cytokines. Results of Combination treatment are displayed on line graph (E) and bar graph (F). Results are given as the means of triplicate measurements ±SD. Similiar results were observed in three independent experiments.

7. The effects of the inhibitory cytokines on NK-92 cell activity and proliferation.

Since TGF-β appeared to play a pivotal role in primary NK cell inhibition, the study proceeded to investigate the inhibition mechanism mediated by TGF-β. To detect the changes of signaling molecule expressions, NK-92 cell was used for experimental convenience. Before the experiments, the cytokine effects on NK-92 cell were checked. NK-92 cells sensitively responded to IL-2. It was observed that TGF-β reduced IFN-γ release in a dose-dependent manner. Meanwhile, IL-10 and IL-4 did not induce significant changes (Figure. 7A). Next, TGF-β, IL-10, and IL-4 effects on cell proliferation were examined. The effects were not clear, as anti-proliferative effects of TGF-β and IL-10 were reversed by dose elevation and IL-4 did not affect proliferation (Figure 7C). As NK-92 cell is the IL-2 dependant cell, we examined the effects of the inhibitory cytokines in absence of IL-2 (Figure 7B, D). Therefore, a higher dose (1 ng/ml) of TGF-β, IL-10, and IL-4 were adjusted than primary human NK cells (10 pg/ml) to induce maximal effect. Although NK-92 cells maintained viability without IL-2 until 48 hours, TGF-β, IL-10, and IL-4 didn't affect IFN-γ secretion and cell proliferation (Figure 7C, D). The expression level of NKp44, NKp46, and NKG2D, which were down regulated in human NK cells, was also measured (Figure 7E). Although the cell lines were stimulated by a higher concentration than primary NK cells, inhibitory effects of TGF-β on the expression of these receptors in NK-92 cells were less than those of primary NK cells. NK cell lines barely responded to IL-10 and IL-4 stimulation

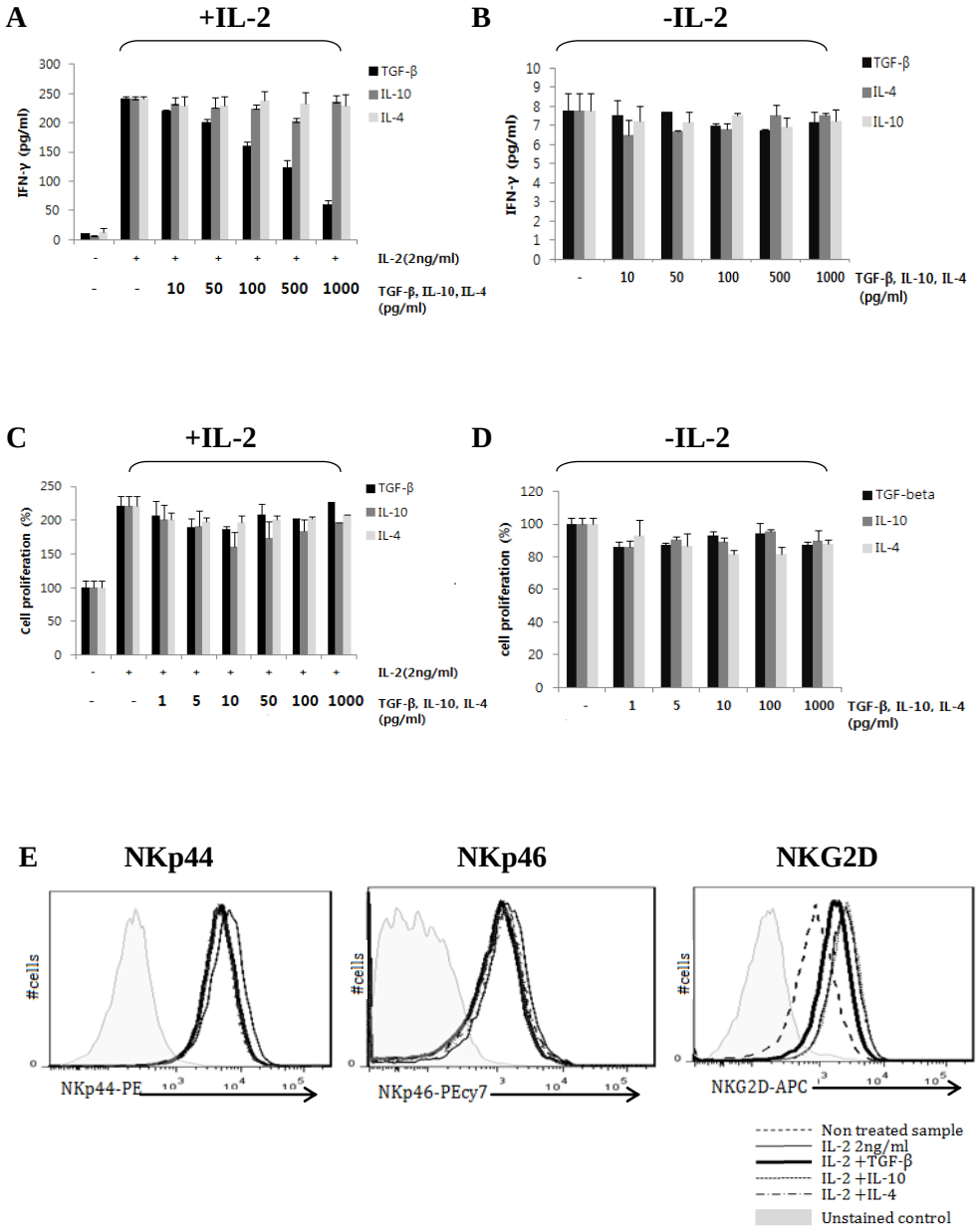
A
+IL-2
IFN-γ (pg/ml)
TGF-β
IL-10
IL-4
IL-2(2ng/ml)
TGF-β, IL-10, IL-4 (pg/ml)
B
-IL-2
IFN-γ (pg/ml)
TGF-β
IL-4
IL-10
TGF-β, IL-10, IL-4 (pg/ml)
C
+IL-2
Cell proliferation (%)
TGF-β
IL-10
IL-4
IL-2(2ng/ml)
TGF-β, IL-10, IL-4 (pg/ml)
D
-IL-2
cell proliferation (%)
TGF-beta
IL-10
IL-4
TGF-β, IL-10, IL-4 (pg/ml)
E
NKp44
NKp46
NKG2D
#cells
NKp44-PE
NKp46-PEcy7
NKG2D-APC
Non treated sample
IL-2 2ng/ml
IL-2 +TGF-β
IL-2 +IL-10
IL-2 +IL-4
Unstained control

Figure 7. The effects of cytokines on NK-92 cell activity and proliferation.

NK-92 cells were cultured with indicated cytokines for 48 hours. (A, B) The culture supernatants were used for IFN-γ detection in a dose-dependent manner (10 pg/ml - 500 pg/ml). (A) shows results of NK-92 cells with 2 ng/ml of IL-2 and the inhibitory cytokine stimulations and (B) shows results of the cells without IL-2. Results are mean ± SD of duplets. (C, D) After incubation, the cells were measured for proliferation in a dose-dependent manner (1 pg/ml - 1000 pg/ml). Data shows results of NK-92 cells with 2 ng/ml of IL-2 (C) and without IL-2 (D). Results are mean ± SD of triplets. (E) The cytokine-treated (2 ng/ml of IL-2 and 1 ng/ml of TGF-β, IL-10, and IL-4) cells were stained by indicated antibodies then analyzed by FACS analysis. Data pooled from three independent experiments are presented.

8. The effects of the inhibitory cytokines on NK-92 cell cytotoxicity.

The effect of TGF-β on the degranulation and target cell killing effect was also evaluated on NK-92 cells. Degranulation was induced with IL-2 in NK-92 cells and IL-2 and TGF-β treated group inhibited IL-2-mediated degranulation, whereas IL-10 and IL-4 did not affect NK cell degranulation (Figure 8A). IL-2-stimulated NK-92 cell displayed 29% cytotoxicity, which was 50% less than human NK cells at 10 : 1 ratio. And TGF- β reduced the cytotoxicity 4% less than IL-2 mediated effect (Figure 8B).

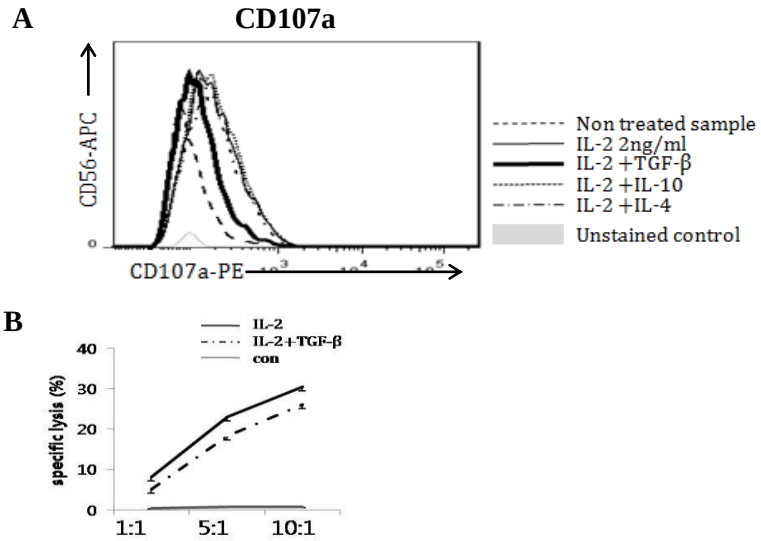

Figure 8. The effects of the inhibitory cytokines on NK-92 cell cytotoxicity.

NK-92 cells were cultured for 48 hours in the medium alone or in the presence of the different combinations of cytokines (2 ng/ml of IL-2 and 1 ng/ml of TGF-β, IL-10, and IL-4). (A) After 4 hour-co-incubation with K-562 cells, NK-92 cells was stained with degranulation markers in response to K-562 cells. (B) NK-92 cell lines were co-incubated with Cr-labeled K-562 cells for 4 hours and the radioactivity was measured. Data pooled from three independent experiments are presented

9. The effects of TGF-β on regulating signal transduction in NK-92 cells.

To elucidate the molecular mechanism of TGF-β-mediated inhibition of IL-2-activated NK cells, It was first checked whether TGF-β inhibit IL-2-mediated signal transduction by reducing IL-2 bound to IL-2 receptor complex. The alpha chain of IL-2 receptor, CD25 is expressed in the activated condition and combines with IL-2 β and γ chain to form a high affinity receptor complex[26]. However, CD25 expression level was not significantly changed neither by IL-2 nor TGF-β in human NK cells. In NK-92 cell lines, CD25 level was enhanced by IL-2 but unaffected by TGF-β (Figure 9A). Next, downstream signaling molecules activated by IL-2 were screened. We found that IL-2-mediated phosphorylation of tyrosine residue of Syk was inhibited by TGF- β (Figure 9B). ZAP 70, one of syk family kinases, plays a critical role in initiating T cell receptor signaling, was not affected by TGF-β stimulation (Figure 9C). Also, IL-2-mediated STAT5 phsophorylation was not affected by TGF-β (Figure 9D). The expression of signaling molecules known to be involved in cytokine release, receptor expression, and cytotoxicty were further screened. However, TGF-β treatment of NK-92 cell lines did not inhibit any of these molecules in the presence or absence of IL-2 (Figure 9D). Phosphorylation of Smad3 was used as a positive control to indicate TGF-β signal worked at the same time point.

Then, the molecules induced with Syk signaling were searched. c-myc has been reported to relate with Syk signaling[27]. As expected, It was observed TGF- β also slightly reduced c-myc expression (Figure 9E, F). These results suggest that TGF-β inhibits IL-2 mediated activation by reducing tyrosine phosphorlyation of Syk and c-myc induction.

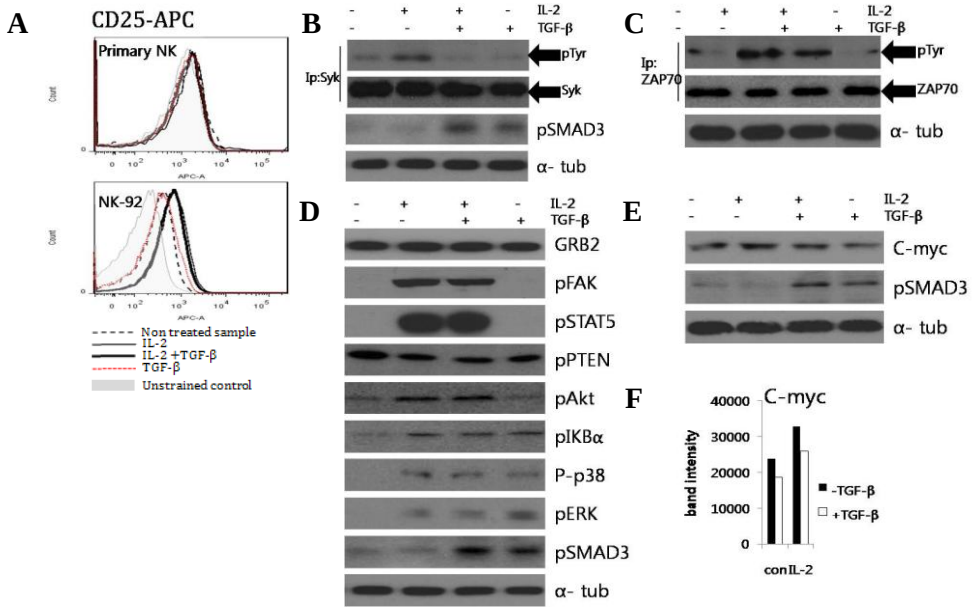

Figure 9. Assessment of signal transduction involved IL-2 signaling in NK-92 cell

(A) Primary NK cells and NK-92 cells were stimulated by 2 ng/ml of IL-2 and 1 ng/ml of TGF-β for 48 hours then analyzed for CD25 expression by FACS. (B-E) NK-92 cells were deprived of serum and IL-2 for 4 hours then treated with or without 2 ng/ml of IL-2 and 1 ng/ml of TGF-β. Cell pellets were collected and analyzed by western blot. (B, C) NK-92 cells were treated with the indicated cytokines for 1 minute, and total cell lysates were immunoprecipitated with anti-Syk (B) and anti-ZAP 70 (C) then blotted with anti-pTyr. The total cell lysates were also detected for phosphorylated Smad3. (D) NK-92 cells were treated with the indicated cytokines for 15 minutes then blotted with GRB2, phosphorylated FAK, STAT5, PTEN, Akt, IKBα, p38, ERK, and Smad3 antibodies. (E) c-myc and pSmad3 expression were measured after 30 minutes stimulation. (F) c-myc quantification was performed by measuring band intensity with ImageJ software. Data pooled from three independent experiments are presented.

10. The effects of TGF-β on transcription factor activity in NK-92 cell.

Even though It was found that TGF-β affected Syk phosphorylation and c-myc expression, there have been few studies of Syk or c-myc related signaling pathway in NK cells. Thus this study was proceeded to lower a level of signal transduction pathway, which is binding activity of transcription factors. The activities of 56 transcription factors in NK-92 cells were detected. Images of the arrays were acquired (Figure 10A), and the data were displayed by quantitative analysis (Figure 10B). The results showed that androgen receptor (AR), CCAAT-displacement protein (CDP), c-myb, GATA, signal transducer and activator of transcription (STAT)-6, STAT-5 were induced more than 600 fold by IL-2, compared to non-treated group. Among them, AR, CDP, and c-myb activity were suppressed upto the basal level by TGF-β, and STAT-5 was down regulated 50% by TGF-β. However, GATA and STAT-6 level were not changed by TGF-β stimulation. Nuclear factor of activated T cells (NFAT-1) was induced 3 fold by IL-2, and decreased 70% (spot density: 3918->2775) by treatment of TGF-β. Activator protein-1 (AP-1), Cyclic AMP-response element binding protein (CREB) increased 1.5 fold by IL-2 treatment. AP-1 was totally suppressed by TGF-β treatment (spot density: 10) and CREB was 50% reduced. Besides, Core Binding Factor (CBF), Estrogen receptor (ER), Forkhead Activin Signal Transducer-1 **(**FAST-1), Myocyte enhancer factor (MEF)-1,-2, Erythroid-specific transcription factor-1,-2 (NF-E1/YY1, NF-E2),Retinoid X receptor(RXR/DR-1), The level of Thyroid hormone receptors (TR/DR-4), Vitamin D receptor (VDR/DR-3), Heat shock element (HSE) and Metal response element (MRE) showed similar to non-treated group and IL-2 stimulated group. With TGF-β treatment, CBF, ER, MRE, and FAST-1 were totally inhibited. RXR/DR-1 level was reduced 30% compared to IL-2 stimulation group. MEF-1, MEF-2, NF-E1, NF-E2, TR/DR-4, VDR/DR-3, and

HSE activity were almost unchanged. E2F-1, EGR, PPAR, Sp-1, STAT-1, and STAT-3 activity were unaffected by any of the cytokine stimulation. In summary, we found that TGF-β inhibited IL-2 induced activation of transcription factors that include AR, CDP, c-Myb, AP-1, CREB, NFAT-1, and STAT-5 (Figure 10C).

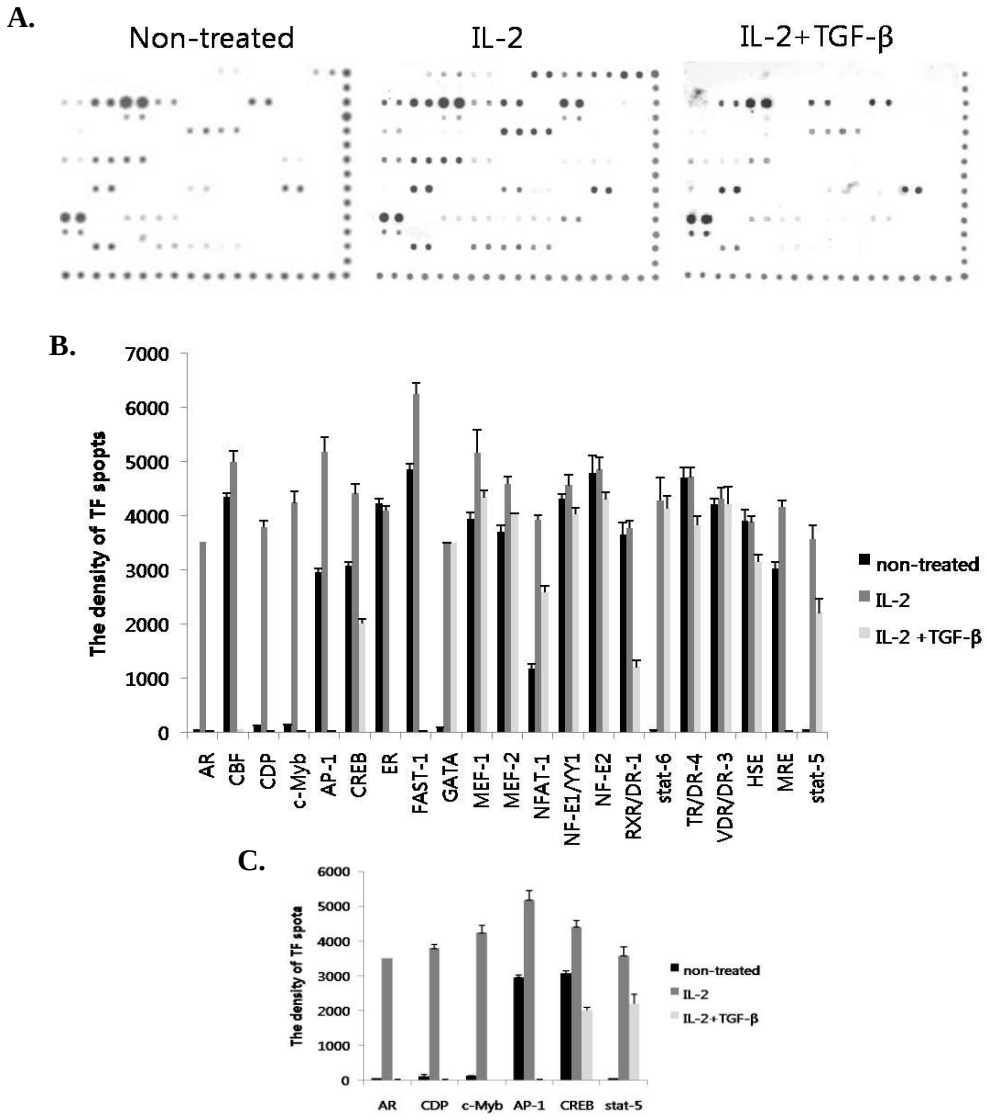

Figure 10. The effects of TGF-β on promoter binding activity in NK-92cell

NK-92 cells were deprived of serum and IL-2 for 4 hours then treated with or without 2 ng/ml of IL-2 and 1 ng/ml of TGF-β for 30 minutes. Nuclear extracts were prepared and used for array assay. (A) The array images were acquired and scanned. (B, C) Quantitative results were displayed by measuring spot intensity with ImageJ software. Results are given as mean ±SD of duplicate measurements.

IV. DISCUSSION

In this study, the single or combination effect of TGF-β, IL-10, and IL-4 (inhibitory cytokines) on human NK cell inhibition was quantitatively compared. Then It was further investigated the molecular mechanism of TGF-β resulting in NK cell inhibition. This study demonstrated that TGF-β was the most potent cytokine on NK cell inhibition even compared with combination treatment of the three cytokines. Also, this study verified that inhibitory effects were more likely to appear in an activating status than in a resting status. Additionally, It was found that TGF-β down regulated Syk tyrosine phosphorylation and c-myc expression. TGF-β appeared to reduce the activities of transcription factors, which are AP-1, c-Myb, CREB, and STAT-5, in NK-92 cell lines. These findings suggest that these signaling molecules and transcription factors are therapeutic targets which may modulate NK cell activity.

Previous studies have demonstrated the effect of the inhibitory cytokines with high concentration at ng range[28], which is much higher than pathological range. Thus study investigated the effect of the inhibitory cytokines at a pathological concentration based on clinical reports (pg range). Although the fact that they exist at a low concentration means several other cytokines intervene, this study is worthy in terms of reproducing the effect of cytokines on NK cells in a pathophysiological conditions. In this study, TGF-β was treated with a much lower dose than the physiological level to compare the effect with those of other cytokines. Nevertheless, TGF-β showed the strongest effect among those cytokines. Therefore, TGF-β may have much stronger effects on NK cell inhibition than our results *in vivo*. Therefore, blocking of TGF-β effects can be a key strategy to keep the activity of NK cells

TGF-β potently inhibited IFN-γ secretion in whole blood, while its inhibitory effect on NK cell appeared to be less than whole blood. Our previous data showed that IFN-γ secretion in whole blood was predominantly mediated by NK cells (unpublished results). Although IFN-γ is mainly secreted from NK cells, crosstalk between various cells in whole blood would trigger synergistic effects upon IFN-γ releasing.

TGF-β and IL-10 reduced NK cell activity, however, combination effect of TGF-β and IL-10 was similar to TGF- β single inhibitory effect. It is known that TGF-β and IL-10 are produced by tumor associated macrophages which provide immunosuppressive microenvironment[29]. TGF-β and IL-10 also induce regulatory T cells that inhibit immune cells[30]. However, the inhibitory mechanism resulting from the TGF-β and IL-10 stimulation is not well known. TGF-β and IL-10 each activates different signaling pathway. TGF-β transduces SMAD pathway and IL-10 transduces JAK-STAT pathway. So if there is no common pathway activated by TGF-β and IL-10 stimulation, co-stimulation may not work synergistically. In the same manner, even though the single effect of IL-10 and IL-4 were not strong, IL-10 and IL-4 combinations mediated addictive effects. Since both IL-10 and IL-4 activate JAK1 phosphorylation, sharing a same signal pathway, and it will induce synergistic effects. And if so, inhibitory molecules sharing the same signal pathway with TGF-β can be a novel therapeutic target.

IL-4 stimulation barely reduced IFN-γ secretion, proliferation, NKp44 expression, and cytotoxicity of NK cells against K562 cells. It demonstrates that IL-4 may not exert serious inhibitory effect at a low dose (10 pg/ml), as represented in Figure 3, although over expressed in tumor microenvironment and known to progress tumor cell survival[31]. Regarding that IL-4 is a regulatory cytokines which counteracts Th1 effects, balancing between the amounts of Th1 cytokine and Th2 cytokine can be a

factor determining NK cell function. Bleotu et al. reported that increased values of IL-4:IL-2 ratio aggravates immunosuppressive state in laryngo-pharyngeal, HPV-positive cancer[32].

TGF-β appeared to cause de-phosphorylation of Syk kinase (Figure 11B). As IL-2 receptor subunits do not possess intrinsic enzymatic activity, they induced protein tyrosine kinase activation by receptor oligomerization[33]. Syk kinase is constitutively bound to serine-rich region of IL-2R β chain and to be activated upon IL-2 stimulation[27]. IL-2 activation of Syk is not fully responsible for IL-2 signaling. Other protein kinases such as Jak and Lck are primarily account for IL-2 signaling[34]. However, TGF-β did not appear to reduce Jak-induced STAT phosphorylation, Lck-induced mitogen-activated protein kinase (MAPK) signaling and NF-κB signaling pathway (Figure 11C). Since Minami Y. et al reported that Syk phosphorylation correlated with c-myc induction[27], we checked c-myc level. As a result, TGF-β also reduced c-myc expression level (Figure 11D). Since c-myc has been reported to highly correlate with cell growth and proliferation[35], data implicates that TGF-β inhibits IL-2 induced NK cell function via reducing viability. It is the first report revealed TGF-β effect on c-myc and Syk level in NK cells.

Although TGF-β suppressed NK cell proliferation and various functions, TGF-β did not affect other signal transduction pathways of NK cells (Figure 11D). Because transcription factor is an endpoint of signal transduction pathways to determine which genes are expressed, the effect of TGF-β on transcription factor activity in NK cells was globally examined (Figure 12). It was demonstrated that IL-2-induced c-Myb, AP-1, CREB, and STAT5 were suppressed upon TGF-β stimulation. This observation is consistent with c-myc inhibition, as these transcription factors also promote cell maturation and proliferation[36]. AP-1 is also related to activation of NK cell function. Nausch N. et al (2006) reported that AP-1 boost NK cell via NKG2D

upregulating and increasing IFN-γ production[37]. Recently, STAT-5 has been reported to have an intrinsic role in mouse NK cell viability and tumor cell control in an Ncr1-Cremouse model[38]. Although this study did not yet verify direct effects of these transcription factors on NK cell function, the data shows that TGF-β blocks NK cell proliferation and activation by hindering several transcription factors' binding activity. This is the first report of profiling TGF-β effects on the activities of transcription factors in NK cells.

In conclusion, the results emphasize significance of TGF-β on NK cell inhibition by comparing with other inhibitory cytokines, and broad investigation about molecular mechanism of TGF-β mediated inhibition of NK cells was firstly conducted. this study provides insights into the effect of TGF-β on the immune modulation of NK cells, and suggests the biomarkers which are susceptible to TGF-β mediated inhibition of NK cells.

V. CONCLUSION

This study demonstrated that TGF-β showed the strongest suppressive effect on IL-2-induced IFN-γ secretion than other inhibitory cytokines (IL-10 and IL-4) in whole blood and human NK cells, whereas IL-4 showed the smallest effect among three cytokines. Also, IL-2-induced TNF-α secretion in whole blood was potently suppressed by TGF-β treatment, being compared with IL-10 and IL-4 treatment. NK cell Proliferation was more suppressed in the group with TGF-β treatment than in IL-10 treated group. IL-4 showed no significant difference. Regarding NK cytotoxicity, the suppressive effect of TGF-β was superior to both IL-10 and IL-4. In addition, TGF-β showed the most potent suppressive effect even compared with the combination effect of the inhibitory cytokines. Hence, these results indicated that TGF-β played a critical role among the inhibitory cytokines in inhibition of NK cell activity.

Next, this study tried to discover the molecular mechanism of the TGF-β induced NK cell inhibition. It was found that TGF-β reduced IL-2-induced Syk tyrosine phosphorylation and c-myc expression. The result suggests Syk and c-myc play an important role in TGF- β-mediated NK cell suppression.

Finally, this study investigated the change of transcription factors triggered by TGF-β in IL-2-activated NK cells. As a result, TGF-β completely inhibited IL-2-induced AR, CDP, c-Myb, AP-1, and CREB activity. TGF-β also partially reduced an activity of STAT-5 and NFAT. Furthermore, TGF-β completely suppressed CBF, ER, FAST-1, and MRE, which are activated regardless of IL-2 stimulation. This results implicate that these transcription factors play a role in TGF-β-induced NK cell inhibition.

Taken together, since the inhibitory cytokines, including TGF-β, are chiefly

involved in NK cell suppression of cancer patients, impeding TGF-β, TGF-β susceptible signaling molecules and transcription factors can be a therapeutic strategy when it comes to promoting NK cell activities.

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<ABSTRACT (IN KOREAN)>

억제성 사이토카인이 NK 세포기능에 미치는 영향에 대한 비교연구

<지도교수 김종선>

연세대학교 대학원 의과학과

이혜미

자연살해세포는 암세포에 대항하여 사이토카인을 분비하거나 직접 죽이는 면역 세포이다. 이에 대응하여 암세포는 억제성 사이토카인을 포함한 억제 환경을 조성함으로써 체내의 면역체계로부터 회피한다.

본 연구에서는 종양세포 주변에 침윤된 자연살해세포가 억제되어 있는 것에 주목하였다. 종양 미세환경에서 가장 강력하게 자연살해세포를 억제하는 조건이 있을 것이라는 가정 하에, 억제성 사이토카인인 TGF-β, IL-10, IL-4를 IL-2에 의해 활성화된 사람의 자연살해세포에 처리하여 억제효과를 비교하였다. 그 결과, TGF-β, IL-10, IL-4는 IFN-γ분비, 증식, NKp44, NKp46, NKG2D 수용체발현, 세포용해과립분비, 종양세포주 살해능력을 억제하였고, NKp30, TRAIL, Fas L, NKG2A, 2B4 발현에는 별다른 영향을 미치지 않았다. 세가지 억제성 사이토카인의 개별효과 비교 시 TGF-β는 가장 강력한 억제효과를 보였고, 병용효과 또한 TGF-β 단독효과에 비해 억제효과가 크지 않았다. 따라서,

TGF-β가 자연살해 세포 억제에 가장 중심적인 사이토카인임을 확인하였다.

다음으로, TGF-β가 NK세포를 억제하는 기전을 분자수준에서 규명하고자 자연살해세포주인 NK-92에 TGF-β처리시에 억제되는 신호전달분자를 찾아보았다. 그 결과 TGF-β를 처리한 그룹에서 Syk의 tyrosine 인산화와 c-myc의 발현이 감소되었음을 확인하였다. 그 외 GRB2의 발현 및 ZAP70, STAT5, p38, ERK, IKBα, FAK의 인산화는 변화하지 않았다. 다음으로 IL-2에 의해 활성화된 NK-92에 TGF-β처리시 변화되는 전사인자의 활성도를 분석하였다. 실험 결과. IL-2에 의해 활성화된 C-Myb, AP-1, CREB, AR 활성도가 TGF-β에 의해 완전히 억제 되는 것을 확인하였다. TGF-β는 또한 IL-2에 의해 야기된 STAT-5와 NFAT의 활성도를 부분적으로 억제하였다. 이 외에도 TGF-β는 IL-2 자극과 무관하게 발현되어 있는 CBF, ER, FAST-1과 MRE의 활성을 완전히 억제하였다. 본 연구결과는 TGF-β가 NK 세포억제에 주요한 영향을 미친다는 것을 비교연구를 통해 증명하였으며, 그로 인해 억제되는 생물학적 마커를 최초로 규명하여 NK세포 억제와 관련된 주요 생물학적 마커를 제시하였다. 이들 연구결과는 NK세포를 이용한 종양면역 치료개발에 적용 가능한 새로운 전략을 고안 하는데 참고 할 수 있을 것이다.

핵심되는 말: 자연살해세포억제, TGF-β, c-myc, Syk, AP-1, CREB, STAT-5,c-Myb