Regulation of natural cytotoxicity receptor (NCR) expression on NK cells

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ABBREVIATIONS

NK cells, natural killer cells

ITAM, Immunoreceptor Tyrosine-based Activating Motif

NCRs, natural cytotoxicity receptors

RT-PCR, reverse transcriptase polymerase chain reaction

PMA, phorbol-12-Myristate-13-Acetate

MAP kinase, mitogen activated-protein kinase

PKC, protein kinase C

HCCs, hepatocellular carcinoma cell lines

TCR, T cell receptor

FasL/ Fas, Fas Ligand/ Fas

TNF/ TNFR, tumor necrosis factor/ tumor necrosis factor receptor

TRAIL/ DR, TNF related apoptosis inducing ligand/ death receptor

MHC, major histocompatibility complex

KIR, killer immunoglobulin-like receptor

p75/AIRM, siglec-7 (sialoadhesin family)

NTB-A, NK, T and B-cell antigen

DNAM1, DNAX accessory molecule-1

LAIR-1, leukocyte-associated Ig-like receptor-1

DAP-12/ DAP-10, DNAX activating protein-12/ 10

PTK, protein tyrosine kinase

ZAP70, 70 kDa zeta associated protein

PI-3 kinase, phosphatidylinositol 3- kinase

IL-2, interleukin-2

ULBP, UL-16 binding protein

MICA/ MICB, major histocompatibility complex (MHC) class I related chain A/ B

HIV-1, human immunodeficiency virus-1

ABSTRACT

Regulation of natural cytotoxicity receptor (NCR) expression on NK cells

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A number of surface receptors expressed on NK cells are related to the regulation of NK cell activity and characterized by either inhibitory or activating properties. Activating receptors usually have short cytoplasmic tails and transduce signals by associating with molecules containing Immunoreceptor Tyrosine-based Activating Motifs (ITAM). Natural cytotoxicity receptors (NCRs) are one major family of activating receptors involved in NK cytotoxicity, and are found only on NK cells. The three family members are NKp46 (NCR1), NKp44 (NCR2) and NKp30 (NCR3). Their surface density might vary with the activation state of NK cells, and the density

may directly correlate with their natural cytotoxicity.

In this study, we investigated the regulation of NCR expression on NK cells and the factors which affect it. We produced stable cell lines expressing full-length NCRs and investigated the change in expression after PMA treatment using flow cytometry, RT-PCR and immunoblotting methods. Expression of NKp30 and NKp46 on Jurkat T cell transfectants appeared to increase by PMA treatment until 8 hr after PMA treatment, but gradually decreased afterward to less than pre-treatment levels. Parallel to surface expression of NCRs, total NCR protein expression also appeared to fluctuate after PMA treatment, but expression of mRNA transcripts was not significantly affected. Experiments with mutant NCR-expressing stable cell lines demonstrated that 288Ser might be critical for NKp46 expression.

In primary NK cells, most cytokines such as IL-2, IL-8, IL-12, IL-15, IL-18, IFN-α1 and IFN-α2b did not appear to significantly alter NCR expression. PD98059, PD150606 and Lactacystin also did not induce any notable changes, suggesting that the MAP kinase and proteosome pathways might not be involved in the regulation of NCR expression. Interestingly, however, PMA slightly down-regulated NKp46

expression on primary NK cells. PMA is a well-known PKC activator. Furthermore, although other PKC inhibitors did not induce or suppress NCR expression, Gö6983, an inhibitor of PKC α, β, γ, δ and ζ, induced a remarkable increase of NCR expression on NK cells. Finally, we show that up-regulation of NCR on NK cells by Gö6983 caused an increase in NK cytotoxicity against hepatocellular carcinoma cell lines (HCCs) and HeLa presumably by increasing granule release.

In conclusion, NCR expression is down-regulated by PMA, a PKC activator, and upregulated by Gö6983, a PKC inhibitor. As a consequence, NK cytotoxicity against HCCs and HeLa appeared to greatly increase after Gö6983 treatment, but slightly decrease after PMA treatment. This suggests that a specific PKC inhibitor, such as Gö6983, could be utilized to enhance NK cytotoxicity and consequently increase host tumor immunity by upregulation of NCR expression.

Key words: Natural killer cell, Natural cytotoxicity receptors, PKC, PMA, Gö6983

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I. INTRODUCTION

Natural killer (NK) cells are a distinct subset of large granular lymphocytes which possess the ability to lyse certain primary tumor cells, tumor cell lines, transplanted allogenic cells and virus-infected cells[1, 2]. NK cells were originally described on the functional basis of their ability to lyse certain tumor cells in the absence of prior antigen stimulation[1]. This lymphocyte subpopulation is characterized by the absence of conventional receptors for antigen, such as surface immunoglobulin or T cell

receptor (TCR), and usually displays a $CD3^-/CD16^+$, $CD56^+$ phenotype[3].

It is well known that target cell lysis by NK cells is primarily mediated by secretory granules which contain perforin and granzymes. Perforin can induce necrosis of target cells and granzymes can induce apoptosis of target cells[4-6]. The nonsecretory/apoptotic pathway which is mediated by the FasL/Fas, TNF/TNF receptor and TRAIL/TRAIL receptor interactions also play an important role in NK cell cytotoxicity[5, 7-11]. NK cells recognize their targets through receptors expressed on the NK surface. Recognition and target cell killing directly induce not only effector mechanisms of the innate immune system but also adaptive immune responses[12].

NK cells express a number of surface receptors and the discovery of an array of the receptors has occurred over the last ten years. In particular, two distinct families of receptors regulate of NK cell activity by binding to ligands which are MHC class I molecules or to still unidentified ligands: the immunoglobulin-like (Ig-like) NK receptors (KIR, NCRs, p75/AIRM1, IRp60, 2B4/CD244, NTB-A, DNAM1/CD226 and LAIR) and the C-type lectin-like NK receptors (CD94/NKG2, NKG2D, NKp80, NKRP1 and the rodent Ly49 receptors)[13-17]. These receptors are characterized by

either inhibitory or activating properties and are involved in the fine regulation of NK cell function. Activating receptors usually transduce signals through association with molecules, such as CD3ζ, FcεRIγ and DAP-12, containing Immunoreceptor Tyrosine-based Activating Motifs (ITAM) that upon phosphorylation transduce an activation signal via cytoplasmic PTKs[14, 18], such as $p72^{syk}$ and ZAP70, in their cytoplasmic tail. Other receptors such as NKG2D associate DAP-10 polypeptide to signal via the PI-3 kinase pathway[19].

NK cells express three different receptors, called Natural Cytotoxicity Receptors (NCRs), which are directly involved in natural cytotoxicity[20]. These include NKp46, NKp44 and NKp30 (also called NCR1, 2 and 3, respectively). A putative NCR should satisfy some of the following requirements and conditions: its expression should be mostly restricted to NK cells; its mAb-mediated crosslinking in a redirected killing assay should trigger NK-cell cytotoxicity; and mAb-mediated masking of the NCR should inhibit the NK-cell-mediated cytotoxicity[18]. Both resting and activated NK cells express NKp46 and NKp30[21, 22], while NKp44 is induced only after in vitro culture with IL-2[23]. NKp46 has an extracellular portion characterized by two C2-type

Ig-like domains[24, 25]. NKp30[22] and NKp44[26, 27] have an extracellular region containing a single Ig-like domain of type V, and NKp44 displays a membrane-proximal region with an extended open conformation typical of hinge-like sequences. NCR transmembrane portions contain positively charged amino acids that are thought to be crucial for their association with CD3ζ or DAP-12[22-24].

NCRs[18] and NKG2D[19] are the major receptors involved in NK cytotoxicity. Human NKG2D has been shown to recognize different ligands, such as MICA and MICB and the family of UL16-binding proteins (ULBP1-4)[19, 28, 29]. Recent studies suggest that membrane-associated heparan sulfate proteoglycans are involved in the recognition of cellular targets by NKp30 and NKp46[30], but other cellular ligands recognized by NCRs have not yet been characterized. NKp46 and NKp44 have also been reported to recognize viral proteins such as influenza virus haemagglutinin and parainfluenza virus haemagglutinin-neuraminidase[31, 32].

Disruption of NCR-ligand interactions by mAb-mediated masking inhibits NK-cell-mediated cytotoxicity. The surface density of NCRs might differ among NK cells and NCR density directly correlates with natural cytotoxicity[18]. Low NCR surface

density is also related to some diseases. For example, NK cells purified from HIV-1-infected patients express significantly decreased levels of NCRs, and this defective NCR expression is associated with a parallel decrease in NCR-mediated killing of different tumor target cells[33].

In contrast to the fundamental structure and function of NCRs, the mechanisms of NCR expression and regulation have been poorly understood. The CD3ζ chain which is associated with NKp30 and NKp46 is not required for NKp46 surface expression in transfected cells[24] and does not appear to be necessary for the surface expression of NKp30[22]. Corticosteroids are known to inhibit NK cell function and reduce the surface expression of activating receptors, particularly NKp46 and NKp30[34], whereas prolactin induces up-regulation of NCR surface expression.

As described above, NK cell activity is primarily regulated by NCR expression[35]. This suggests that NK cytotoxicity could be enhanced by increasing NCR surface expression. For this purpose, it is necessary to understand how NCR expression is regulated in NK cells. In this study, we first investigated which factors affect NCR expression on primary NK cells. We also investigated how NCR expression is

regulated by such factors in NCR-transfected Jurkat T cells and in primary NK cells.

Finally we investigated how NK cytotoxicity is correlated with NCR expression.

II. MATERIALS AND METHODS

1. NK cell preparation NK cells were purified from the whole blood of healthy volunteers by negative selection using the RosetteSep™ NK enrichment antibody cocktail (StemCell Technologies Inc, Vancouver, Canada) as previously described[36]. Briefly, 1 ㎖ of whole blood was mixed with 50 ㎕ of RosetteSep™ NK enrichment cocktail, and incubated for 20 min at room temperature. The blood sample was then diluted with an equal volume of phosphate buffered saline (PBS, pH7.4) containing 2% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA). The diluted sample was layered on to Ficoll-paque and centrifuged at 1600 rpm for 20 min at room temperature. The NK cell layer was collected and the enriched NK cells were washed three times with PBS containing 2% FBS. Purified NK cells were >80% $CD56^{+}CD16^{+}/CD3^{-}$ (Beckman Coulter, Fullerton, USA) and were maintained in RPMI 1640 media containing 10% FBS and 100 unit/㎖ of recombinant IL-2 (Endogen, Woburn, MA, USA).

2. Cell lines and cell culture Hepatocellular carcinoma cell lines HepG2 (ATCC HB 8065) and Hep3B (ATCC HB 8064) were used as target cells and maintained in

MEM containing 10% FBS (Gibco BRL). Jurkat T lymphoma cell line (ATCC TIB 152) was cultured in RPMI1640 containing 10% FBS. HeLa (ATCC CCL 13) was cultured in DMEM containing 10% FBS.

3. Construction of expression vectors for NCRs A series of NCR constructs were generated by PCR amplification of the NCR gene with specific primer sets described below. The NKp46 coding region was amplified using the upper: 5'- TATA CGGAATTCATGTCTTCCACACTCCCTGCC-3' and lower: 5'-GACACCAAGCTT TCAAAGAGTCTGTGTGTTCAGCCTTCT-3' primers, containing the underlined EcoRI and HindIII sites, respectively. The NKp30 coding region was amplified using upper: 5'-ATCAATGAATTCATGGCCTGGATGCTGTTGCTCATC-3' and lower: 5'-GCCTTTAAGCTTCTAGGGACATCTGGGCTCTGGAATCAC-3' primers containing the underlined EcoRI and HindIII sites, respectively. Amplified DNAs were gel purified, digested with the appropriate enzymes, and ligated into the pcDNA3.1 *myc*-His (-) A mammalian expression vector (Invitrogen, Carlsbad, CA, USA) that had been digested with the appropriate restriction enzymes. A series of NCR point mutant constructs were generated by quick change site-directed mutagenesis with specific

primer sets described below. NKp46 S279A upper: GTTGAAGACTGGCTCGCCAG GAAGAGGACTAGA and lower: TCTAGTCCTCTTCCTGGCGAGCCAGTCTTCA AC; NKp46 T283A upper: CTCAGCAGGAAGAGGGCCAGAGAGCGAGCCAGC and lower: GCTGGCTCGCTCTCTGGCCCTCTTCCTGCTGAG; NKp46 S288A upper: ACTAGAGAGCGAGCCGCCAGAGCTTCCACTTGG and lower: CCAAGT GGAAGCTCTGGCGGCTCGCTCTCTAGT; NKp46 S291A upper: CGAGCCAGC AGAGCTGCCACTTGGGAAGGCAGG and lower: CCTGCCTTCCCAAGTGGCA GCTCTGCTGGCTCG; NKp46 T292A upper: GCCAGCAGAGCTTCCGCCTGGG AAGGCAGGAGA and lower: TCTCCTGCCTTCCCAGGCGGAAGCTCTGCTGG C; NKp30 S176A upper: GGAACACACTGCCACGCCTCAGATGGGCCCCGA and lower: TCGGGGCCCATCTGAGGCGTGGCAGTGTGTTCC; and NKp30 tyrosine motif deletion mutant upper: GCCGTGGGCAGCACCGTCAAATGCCACTGTCAC ATG and lower: CATGTGACAGTGGCATTTGACGGTGCTGCCCACGGC. All constructs were confirmed by DNA sequencing.

4. Stable cell lines Constructed wild-type and mutant NCR expression vectors were transfected into Jurkat T cells by electroporation. Cells were harvested and

resuspended in ice-cold PBS and 0.8 ㎖ aliquots were transferred into electroporation cuvettes (Bio-Rad Laboratories, Hercules, CA, USA). DNA (10 ㎍) was added to the aliquots, cuvettes were placed in the holder of a MicroPulser electroporation apparatus (Bio-Rad) and shocked at 0.25 ㎸ initial voltage and 960 ㎌ capacitance. Cells were diluted 20-fold into complete media without antibiotics and cultured for 48 hr. After 2 days, the cells changed to complete selection media with antibiotics containing 1 ㎎/㎖ G418 (Duchefa, Haarlem, Netherland). NCR and mutant NCR expression were confirmed by flow cytometry.

5. Flow cytometric analysis of NCRs Cell surface NCRs were quantified by flow cytometric analysis. NK cells were washed twice with ice-cold PBS containing 0.05% BSA. Cells were incubated with PE labeled anti-NKp30 or anti-NKp46 antibody (Beckman Coulter) for 30 min at 4℃. After two washes with 0.05% BSA-PBS, cells were analyzed using a FACScalibur flow cytometer (Becton Dickinson Bioscience, Lincoln Park, NJ, USA).

6. RT-PCR analysis of NCRs Total RNAs was extracted from NK cells and tumor cell lines using an RNAeasy Kit (Qiagen, Santa Clara, CA, USA). The integrity of

isolated total RNA was confirmed by 1.5% agarose gel electrophoresis. To synthesize cDNA, 1 ㎍ of each RNA sample was mixed with 100 ng random hexamer, 6 ㎕ of 5X first strand buffer, 12 ㎕ of 2.5 mM dNTPs (TaKaRa, Shiga, Japan) and 200 units of murine Molony leukemia virus reverse transcriptase (MMLV-RT) (Invitrogen) and incubated at 42℃ for 80 min. The reaction mixture was boiled at 95℃ for 5 min, quickly chilled on ice, then used for PCR without further manipulation. The PCR reaction mixture was prepared with 2.5 ㎕ of cDNA, 2 ㎕ of 2.5 mM dNTPs, 20 pmol primer, 2.5 ㎕ of 10X PCR buffer, 13.8 ㎕ of distilled water and 1 unit of *Taq* polymerase (TaKaRa). PCR reactions were performed with the appropriate primers (used in the construction of NKp30 and NKp46 expression vectors).

7. Western Blot analysis of NCRs Target cells were lysed with lysis buffer (10 mM Tris-HCL, pH7,4 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1mM PMSF, 15 ㎍/㎖ leupeptin, 2 mM NaF, 2 mM $NaVO_4$). Lysates were separated on SDS-polyacrylamide gels and transferred to PVDF membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Membranes were blocked with 5% BSA in PBS

containing 0.1% Tween-20 (PBST) for 2 hr, incubated with anti-NKp30, anti-NKp46 (R&D Systems, Minneapolis, MN, USA) and α-tubulin (Sigma Chemical Co., St. Louis, MO, USA) antibodies for 4 hr and washed with PBST. The membranes were then incubated with peroxidase-conjugated affinipure donkey anti-goat IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 2 hr, and washed with PBST. Blots were visualized with Supersignal WestDico chemiluminescent substrate (Pierce, Rockford, IL, USA).

8. NK cytotoxicity assay: The JAM test NK cell-mediated apoptotic target cell death was measured using the JAM test. For labeling, 2×10^4 cells were incubated with 20 μCi of [^{3}H]-thymidine (37 MBq/ml, NEN, Boston, MA, USA) for 20 hr at 37℃ in 96-well microtiter plates, then washed three times with culture media without 10% FBS. [^{3}H]-thymidine labeled target cells and NK cells were mixed at the indicated ratios. After a 2 hr incubation, cells and media were aspirated onto glass fiber filters (size 90 x 120 mm) using a semiautomated 96 well harvester (TOMTEC, Hamden, CT). The filters (Wallac Oy, Turkr, Finland) were washed, dried, and sealed with melt-on scintillator sheets (Wallac), and radioactivity was measured with a beta

counter (Wallac). Percentage of apoptotic cell death was calculated by the following formula: % DNA fragmentation = [1-(experimental value / control value)]*100. The control value was determined by incubating target cells in culture medium alone. Data are presented as the mean of at least three independent experiments.

9. NK cytotoxicity assay: ^{51}Cromium release assay NK cell-mediated target cell killing was assessed using a standard ^{51}Cr release assay. For labeling, 3×10^3 cells were incubated with 10 μCi of ^{51}Cr (NEN, Boston, MA) for 60 min at 37℃ in 96-well microtiter plates, then washed three times with culture media without 10% FBS. ^{51}Cr-labeled target cells and NK cells were mixed at the indicated effector to target (E:T) ratio. After 4 hr of coculture with NK cells, cell-free supernatant was collected and radioactivity was measured with a gamma counter. Percentage of specific ^{51}Cr release was calculated by the following formula: % cytotoxicity = [(experimental ^{51}Cr release - spontaneous ^{51}Cr release) / (maximum ^{51}Cr release - spontaneous ^{51}Cr release)] x 100. For control experiments, target cells were incubated either in culture medium alone to determine spontaneous release or in a mixture of 2% Triton X-100 to define maximum ^{51}Cr release. Data are presented as the mean of at least three independent experiments.

10. Fixation of NK cells For blocking granule release, NK cells were incubated with RPMI 1640 containing 0.5% paraformaldehyde for 20 min and washed twice with PBS. The concentration of paraformaldehyde and incubation time was minimized to avoid adverse effects as much as possible.

III. RESULTS

1. NCR constructs and stable cell lines

To investigate how NCR expression is regulated, expression vectors were constructed and transfected into the Jurkat T lymphoma cell line. Since NCR expression is restricted to NK cells, we chose Jurkat T cells for transfection and expression. Full-length NKp46 (NCR1) and NKp30 (NCR3) genes containing the extracellular domain, transmembrane domain and cytoplasmic tail portion were inserted into the pcDNA3.1/*myc*-His (-) A expression vector (Invitrogen) (Fig. 1A). Expression vectors were transfected using an electroporation apparatus, and the transfected cells were selected in G418 media. NKp30 and NKp46 expression clones (4 clones each) are shown in Fig. 1B. Of these clones, NKp30 1F7 and NKp46 4E4 were used for the following experiments.

2. Regulation of NCR expression by PMA in stable cell lines

Using the stably transfected cell lines above, we first investigated the effect of PMA treatment on NCR expression. PMA is a PKC activator[37]. PMA increased the

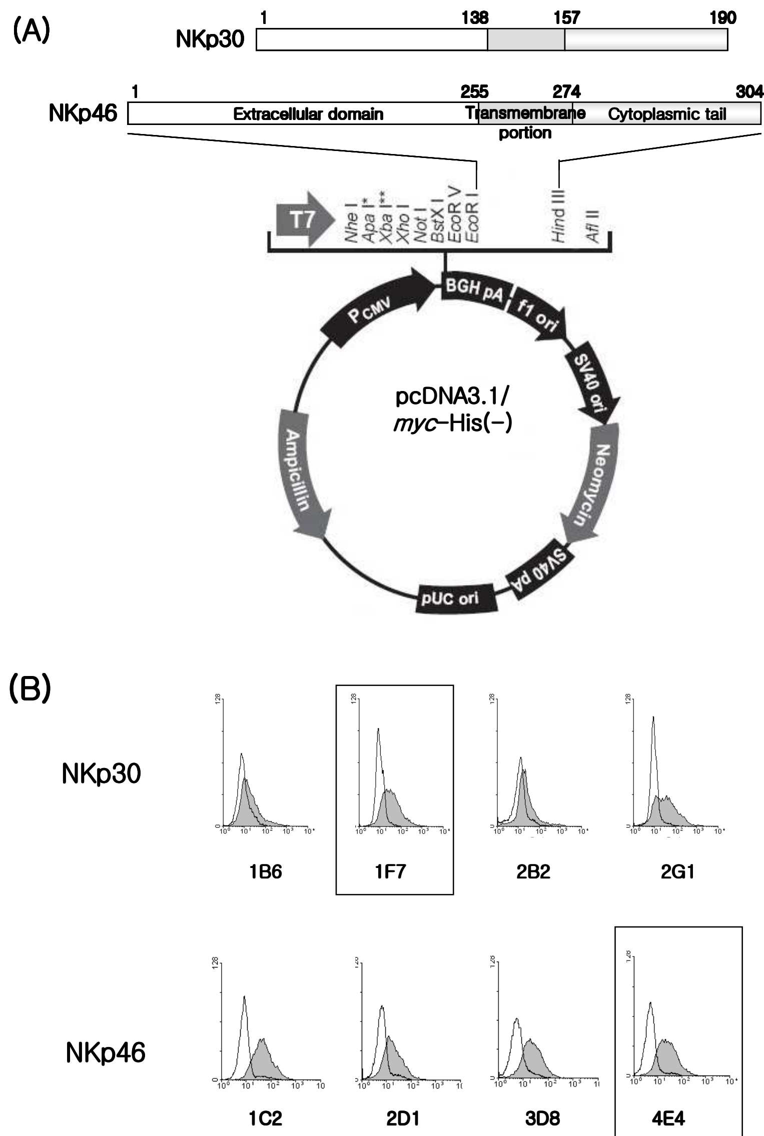
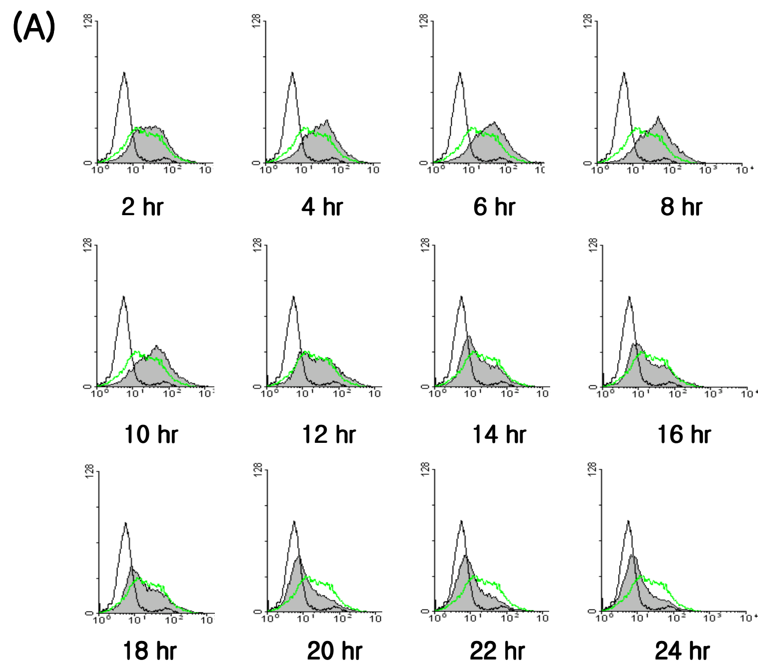
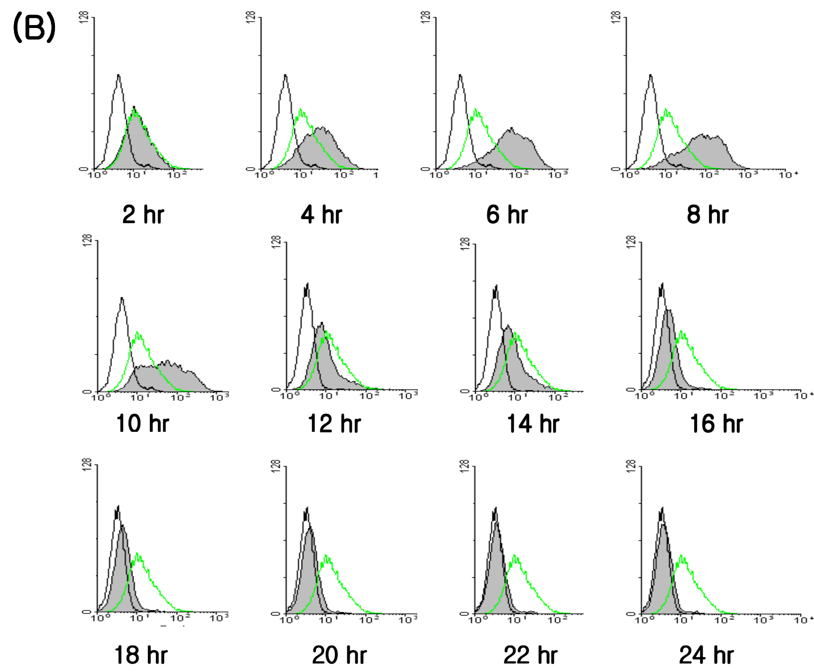


Fig. 1 NCR constructs and stable cell lines (A) Schematic diagram of NCR constructs. NKp30 and NKp46 full length PCR products were inserted between EcoRI and HindIII enzyme sites of pcDNA3.1 *myc*-His (-) vector. (B) NKp30 and NKp46 stable cell lines. Some clones expressing NCRs on their surface were selected through determining by flow cytometry analysis. (Black lines: isotype control, Filled: NCR expressions)

expression of NKp30 on the 1F7 stable cell line (Fig. 2A). Interestingly, NKp30 expression on 1F7 slightly increased until 8 hr and decreased gradually after that. NKp46 expression on the 4E4 clone showed a similar phenomenon after PMA treatment and maximum expression occurred around 6~8 hr after PMA treatment (Fig. 2B). Both NKp30 and NKp46 expression levels were lower than pre-treatment levels after 24 hr. NCR expression levels as a function of time are shown using a relative MFI ratio which represents the ratio of mean experimental value to mean base expression value (Fig. 2C). The relative MFI ratio shows the same results as the histogram: NCR expression on stable cell lines gradually increases to a maximum level, then decrease to levels lower than the initial values (Fig. 2C).

NCR mRNA expression was measured by RT-PCR and using GAPDH as the internal control. Unlike the surface expression, NKp30 and NKp46 transcript expression were not significantly changed after PMA stimulation (Fig. 3). This suggests that NCR expression in Jurkat transfectants is regulated by PMA at the post-transcriptional level.


(A)
2 hr
4 hr
6 hr
8 hr
10 hr
12 hr
14 hr
16 hr
18 hr
20 hr
22 hr
24 hr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22 hr
24 hr

(C)

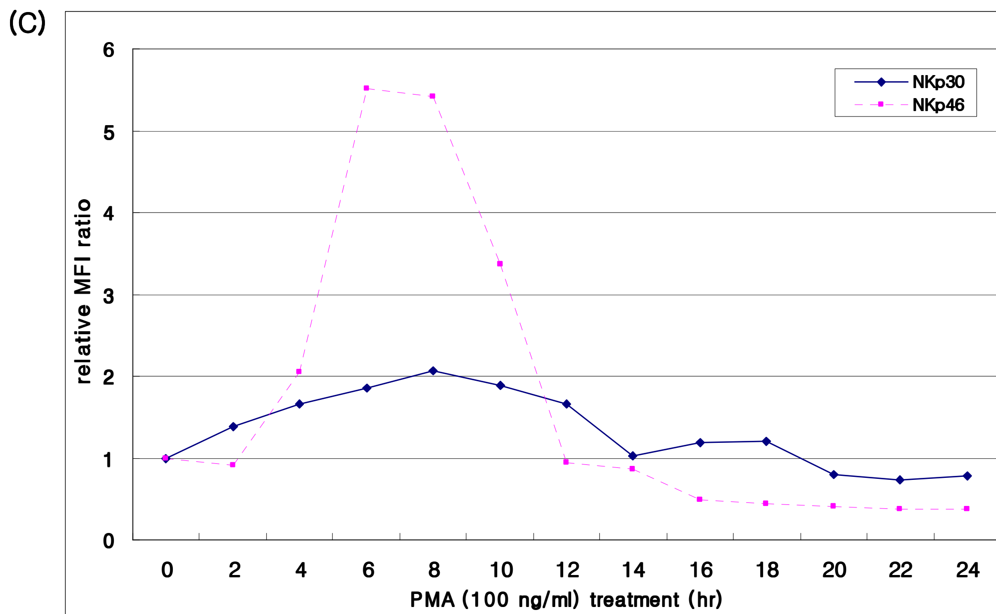


Fig. 2 Regulation of NCR expression by PMA (A) NKp30 and (B) NKp46 surface expression on stable cell lines were determined by flow cytometry. (Black line: Isotype control, Gray line: None, Filled: NCR expression after PMA (100 ng/mℓ) treatment) (C) The NCR expressions were presented by relative MFI ratio.

Total NCR protein expression in stable cell lines was investigated using immunoblotting. Although mRNA expression of NKp30 and NKp46 were not changed after PMA stimulation, total protein levels fluctuated as a function of time (Fig. 4). In general, the blotting results were similar to those of flow cytometry analysis (Fig. 2). Total NCR protein expression after PMA stimulation also increased until 6~8 hr and then gradually decreased (Fig. 4).

3. Identification of the sequence motif which affects NCR expression

To identify the sequence motif which affects NCR expression, we constructed expression vectors of NCR point mutants. We altered serine and threonine sites which are expected to be PKC binding sites to alanine: Ser176 in the cytoplasmic tail of NKp30, Ser279, Thr283, Ser288, Ser291 and Thr292 in the cytoplasmic tail of NKp46 (Fig. 5A). Mutant NCR expression vectors were transfected to Jurkat T cells by electroporation and the transfected cells were cultured in complete media containing G418 neomycin. Mutant clones were selected by flow cytometric analysis, and NKp30 S176A 3F2, NKp46 S279A 3A8, NKp46 T283A 4E7, NKp46 S288A 4C5, NKp46 S291A 5D7 and NKp46 T292A 4C1 clones were used in the following experiments (Fig. 5). These mutant proteins were well expressed on the surface of Jurkat T cells, although NKp46 S288A was much less expressed than wild type NKp46 (Fig. 5B). Regulation of mutant NCR expression after PMA stimulation was assessed by flow cytometry and the histogram represents the change in NCR surface expression on mutant clones cultured with PMA for 12 hr and 24 hr (Fig. 5C). Like wild type NKp30 and NKp46, expression of NKp30 S176A, NKp46 S279A, NKp46

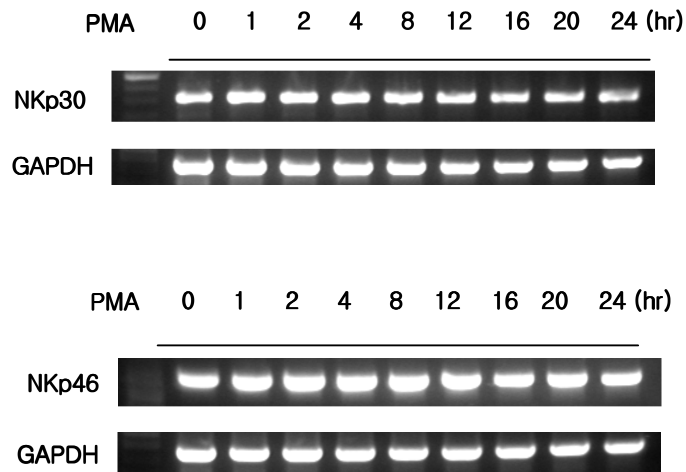


Fig. 3 Transcription of NCR mRNAs in stable cell lines Expression of the NCR mRNAs in NCR stable cell lines after PMA (100 ng/㎖) treatment for indicated times were determined by RT-PCR.

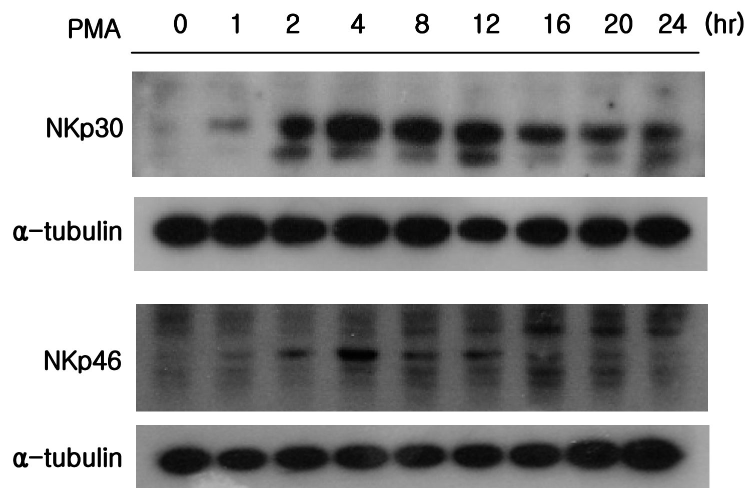
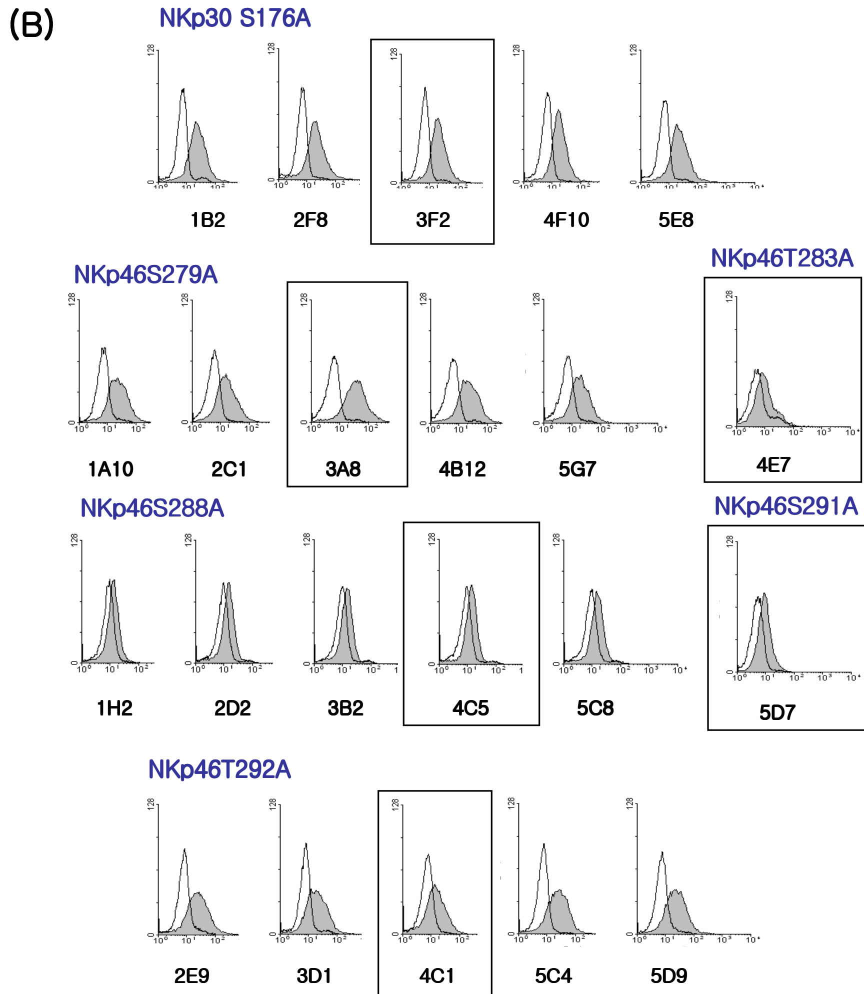


Fig. 4 NCR total protein expressions in stable cell lines Expressions of the NCR total proteins in stable cell lines after PMA (100 ng/㎖) treatment were determined by Immunoblotting. Internal control was set by α-tubulin.

(A)
NKp30
158
190
STVYYQGKCHCHMGTHCHSSDGPRGVIPEPRCP
S176A
A
NKp46
275
304
EDWLSRKRTRERASRASTWEGRRRLNTQTL
S279A
A
T283A
A
S288A
A
S291A
A
T292A
A


(B)
NKp30 S176A
1B2
2F8
3F2
4F10
5E8
NKp46S279A
1A10
2C1
3A8
4B12
5G7
NKp46T283A
4E7
NKp46S288A
1H2
2D2
3B2
4C5
5C8
NKp46S291A
5D7
NKp46T292A
2E9
3D1
4C1
5C4
5D9

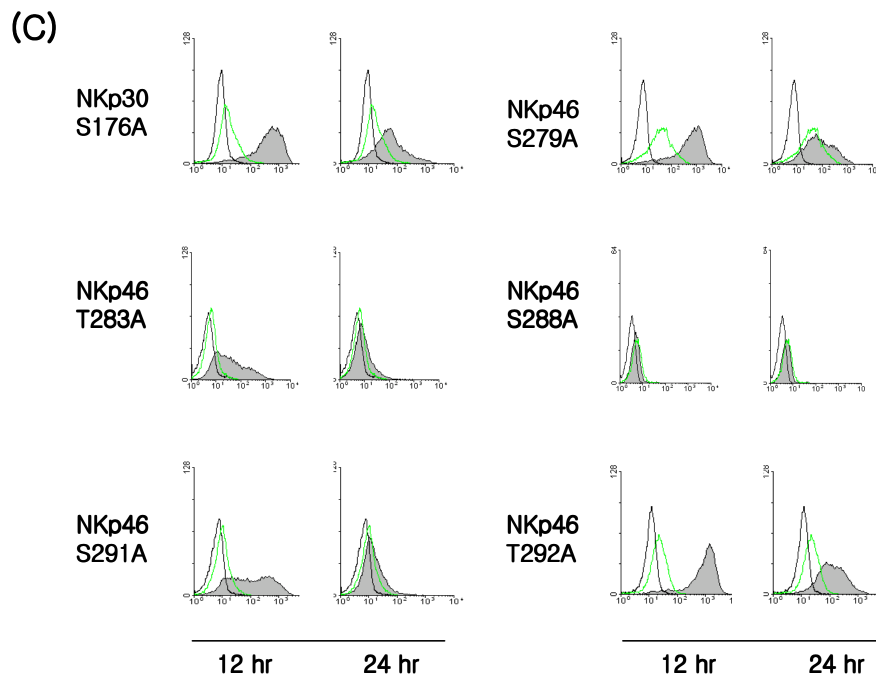


Fig. 5 NCR mutant constructs and stable cell lines expressing them (A) NCR mutant constructs were shown by schematic diagrams. (B) NCR mutant constructs were transfected to Jurkat cell lines and some stable clones were selected by flow cytometry. (C) The histograms showed the expressions of NCR mutants after PMA (100 ng/㎖) treatment.

T283A, NKp46 S291A and NKp46 T292A mutant proteins increased 12 hr after PMA stimulation, but decreased after 24 hr. However, expression of the NKp46 S288A mutant was not affected by PMA stimulation (Fig. 5C). This suggests that Ser288 of NKp46 is important for the regulation of surface expression of NKp46 by PMA.

4. Effect of cytokines on primary NK cell NCR expression

Since NCR expression in stable cell lines was regulated by PMA at the post transcriptional level, we next investigated how NCR expression is regulated in primary NK cells. As a first step, we screened various factors that are known to affect the expression of NCR on primary NK cells. To discover factors which increase NCR expression on NK cells, we tested several cytokines which are known activators of immune cells. IL-2, IL-8, IL-12, IL-15, IL-18, IFN-α1 and IFN-α2b did not significantly change NKp30 and NKp46 expression on primary NK cells (Fig. 6). Histograms and relative MFI ratios showed only unchanged or slightly altered NCR expression (Fig. 6A, B).

5. Regulation of NCR expression by PMA in primary NK cells

We next investigated the effects of PMA, which altered of NCR expression in stable cell lines, and IL-2, which is necessary for primary NK cell growth, in more detail. NKp30 expression on primary NK cells is represented by histogram appeared to increase a little after addition of PMA to the culture (Fig. 7A). IL-2 treatment, however, did not appeared to increase NKp30 expression compared to untreated cells

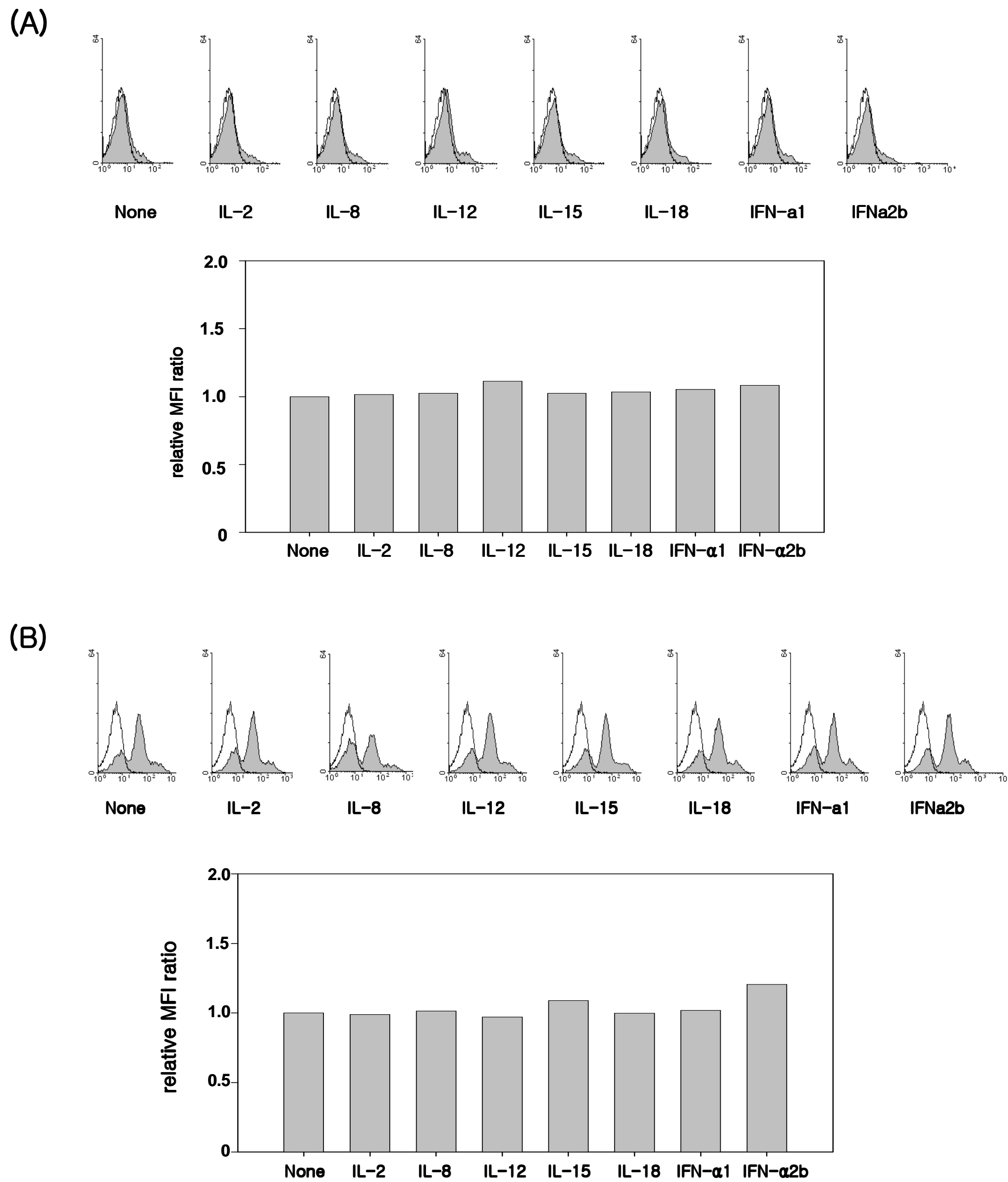


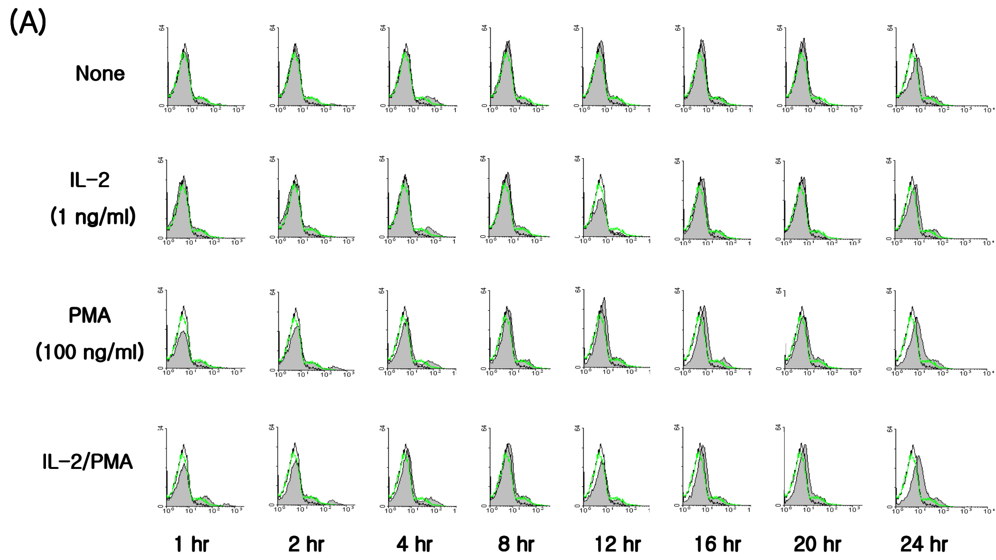
Fig. 6 Effects of cytokines upon NCR expression on NK cells (A) NKp30 and (B) NKp46 expressions on primary NK cells after cytokine treatment were determined by flow cytometry and presented by relative MFI ratio. (Black line: Isotype control, Filled: NCR expression)

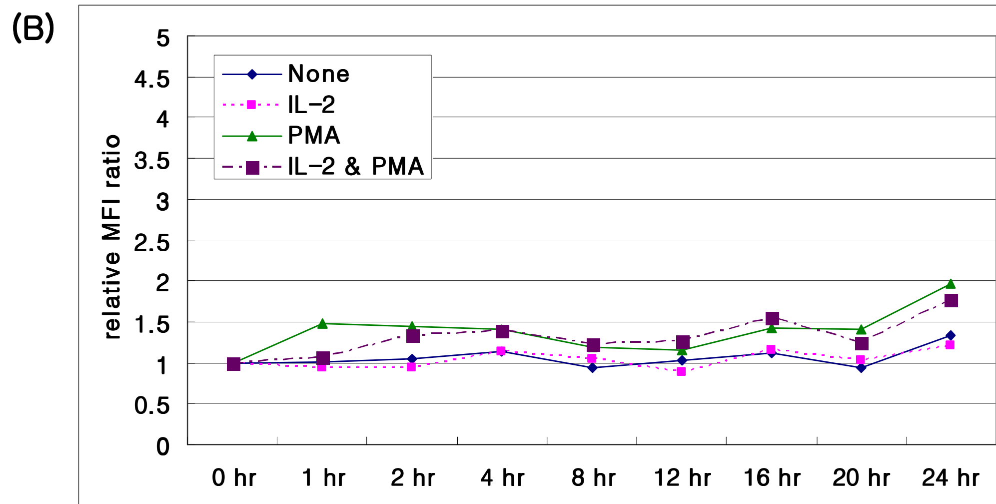
(Fig. 7A). Plots of Relative MFI values show that NKp30 expression is slightly increased by PMA, but not affected by IL-2 (Fig. 7B). Unlike NKp30, NKp46 expression on NK cells increased in untreated cultures, but IL-2 did not change of expression. Moreover, PMA appeared to reduce the expression of NKp46 on primary NK cells compared to untreated control group cells (Fig. 7C). By relative MFI value, PMA diminished the expression of NKp46, while IL-2 did not significantly affect it (Fig. 7D). NCR mRNA expression in PMA-treated primary NK cells was measured by RT-PCR, with GAPDH as internal control. Unlike the stable cell lines, NKp30 and NKp46 transcript expression in primary NK cells paralleled surface expressions (Fig. 7E).

We next tested inhibitors of MAP kinase kinase (MEK), calpain and proteosomes (PD98059, PD150606 and Lactacystin, respectively). None of the selected inhibitors induced any particular change in NCR expression on primary NK cells (Fig. 8).

6. Regulation of NCR expression by PKC inhibitors in primary NK cells

Since PMA (a PKC activator) appeared to be more effective in regulating NCR


(A)
None
IL-2
(1 ng/ml)
PMA
(100 ng/ml)
IL-2/PMA
1 hr
2 hr
4 hr
8 hr
12 hr
16 hr
20 hr
24 hr


(B)
relative MFI ratio
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
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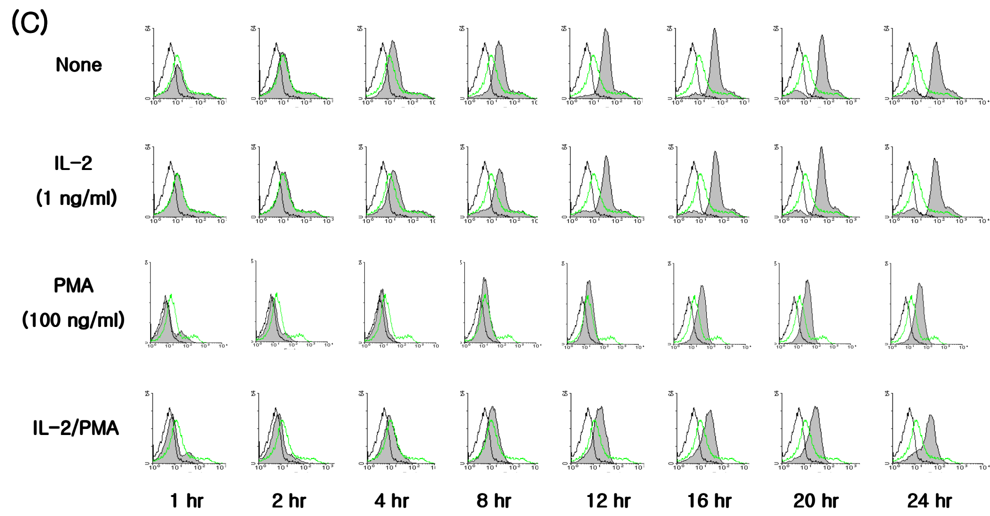
IL-2
PMA
IL-2 & PMA
0 hr
1 hr
2 hr
4 hr
8 hr
12 hr
16 hr
20 hr
24 hr


(C)
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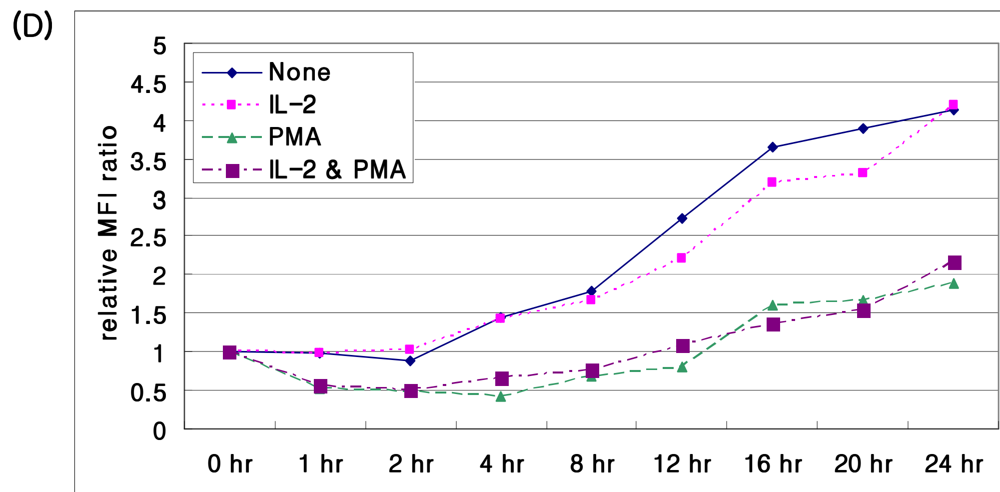
IL-2
(1 ng/ml)
PMA
(100 ng/ml)
IL-2/PMA
1 hr
2 hr
4 hr
8 hr
12 hr
16 hr
20 hr
24 hr


(D)
relative MFI ratio
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
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IL-2
PMA
IL-2 & PMA
0 hr
1 hr
2 hr
4 hr
8 hr
12 hr
16 hr
20 hr
24 hr

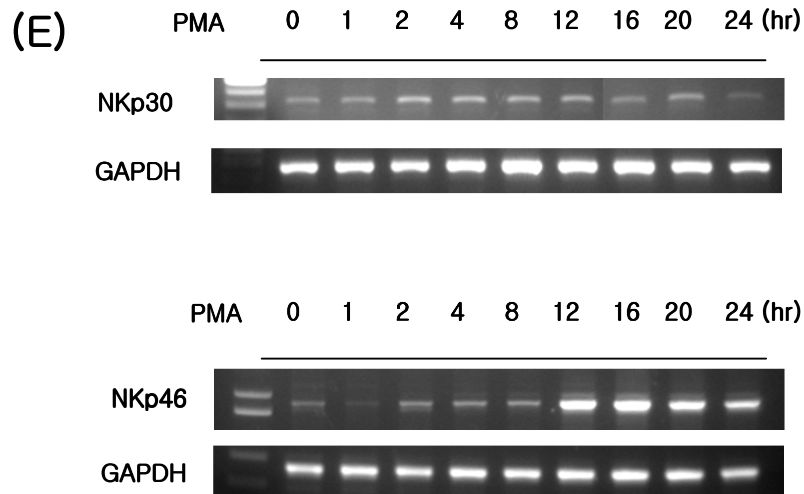


Fig. 7 Regulation of NCR expression upon NK cell activation by IL-2, PMA, and LPS (A) NKp30 and (C) NKp46 surface expression on primary NK cells after indicated treatment for 24 hr. The histogram data were represented by relative MFI ratio (B, D). (E) Expression of the NCR mRNAs in primary NK cell after PMA (100 ng/㎖) treatment for indicated times were determined by RT-PCR. (Black line: Isotype control, Gray line: none, Filled: indicated treatment)

expression than any other cytokine or inhibitor tested, we next investigated the effects of PKC inhibitors Gö6976, Gö6983, Rottlerin and Bisindolylmaleimide III (BisIII) on NCR expression. Interestingly, Gö6983 induced a dramatic increase in NKp30 expression on primary NK cells, while the others did not (Fig. 9A). The relative MFI ratio of Gö6983 treated NK cells was about 20-fold higher than that of the controls, while NK cells treated with the other PKC inhibitors had ratios similar to

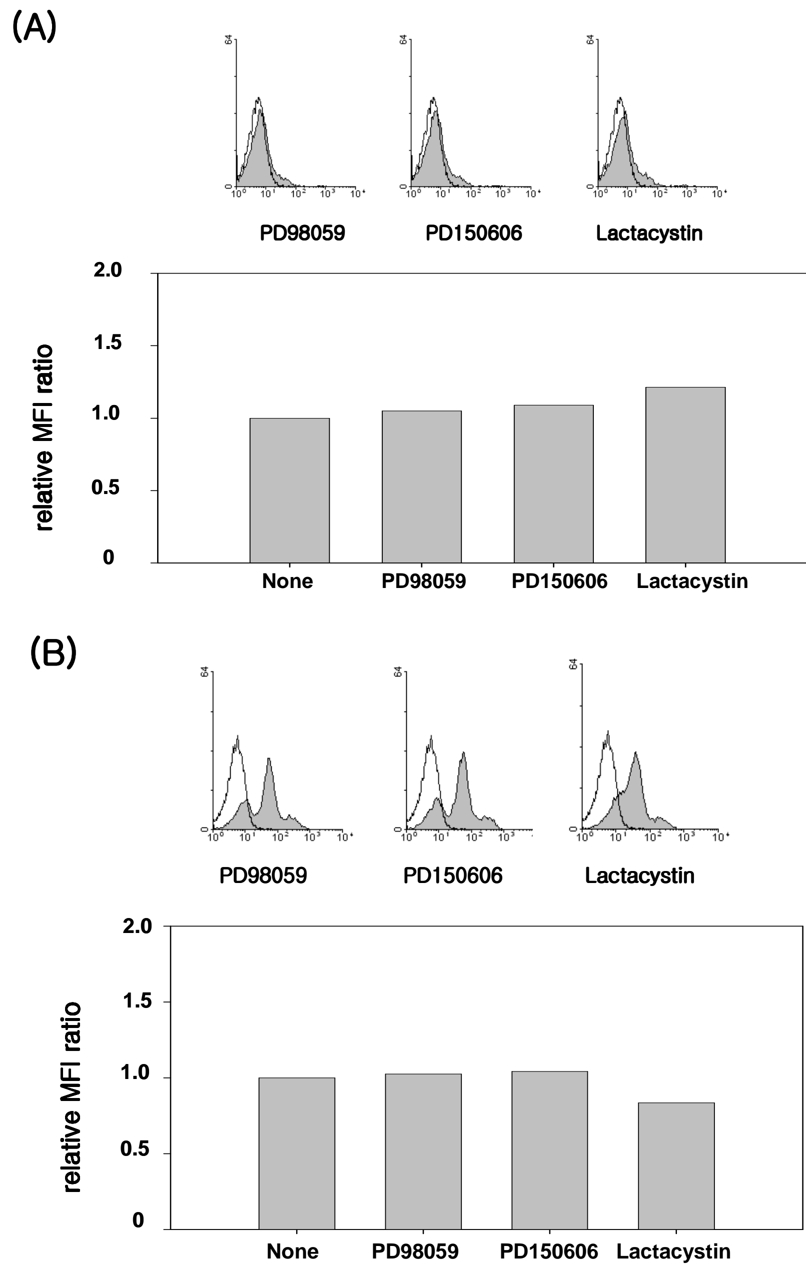


Fig. 8 Effects of other inhibitors upon NCR expression (A) NKp30 and (B) NKp46 expressions on primary NK cells were determined by flow cytometry and presented by relative MFI ratio. The inhibitors were treated in 5 μM for 24 hr. (Black line: Isotype control, Filled: NCR expression by indicated treatment)

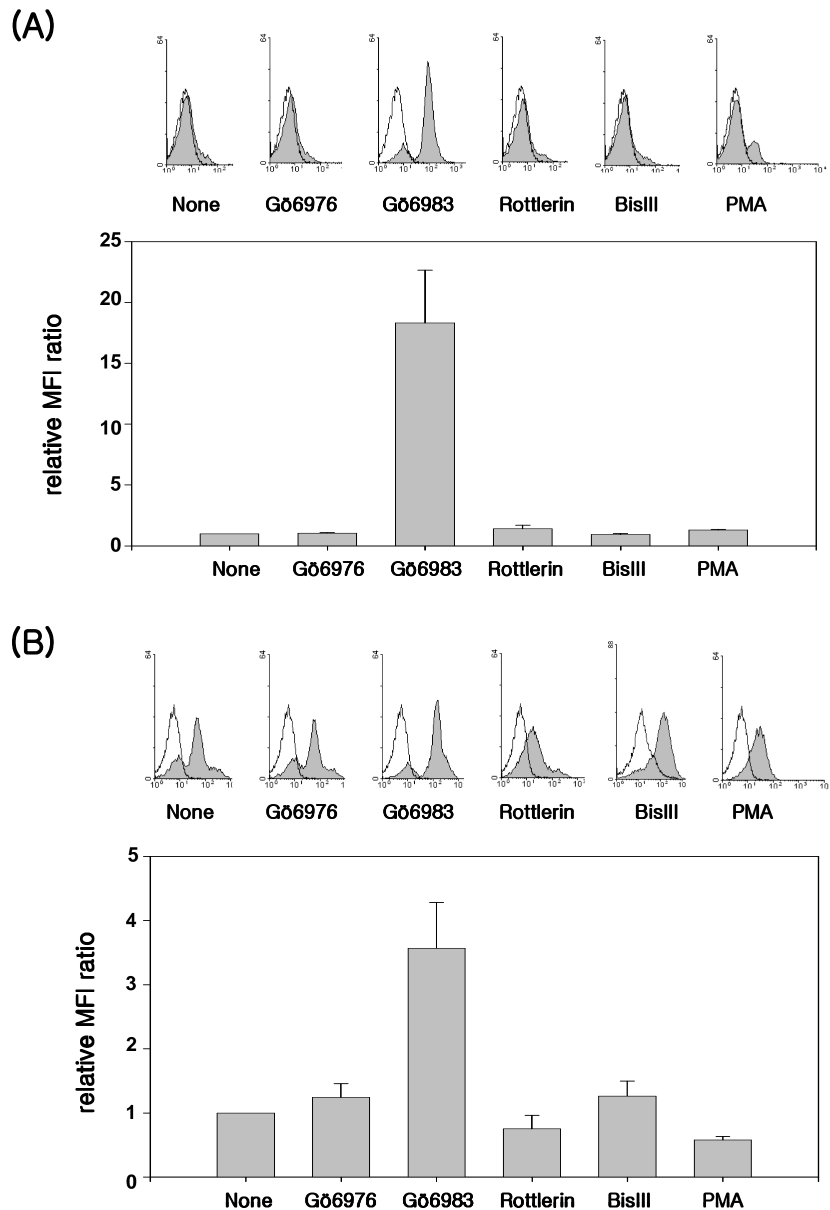


Fig. 9 Effects of PKC inhibitors upon NCR expression in primary NK cells (A) NKp30 and (B) NKp46 surface expressions were determined by flow cytometry and presented by relative MFI ratio. The inhibitors were treated in 5 μM for 24 hr. (Black line: Isotype control, Filled: NCR expression by indicated treatment) The data are presented as a mean of at least three independent experiments (mean±SD).

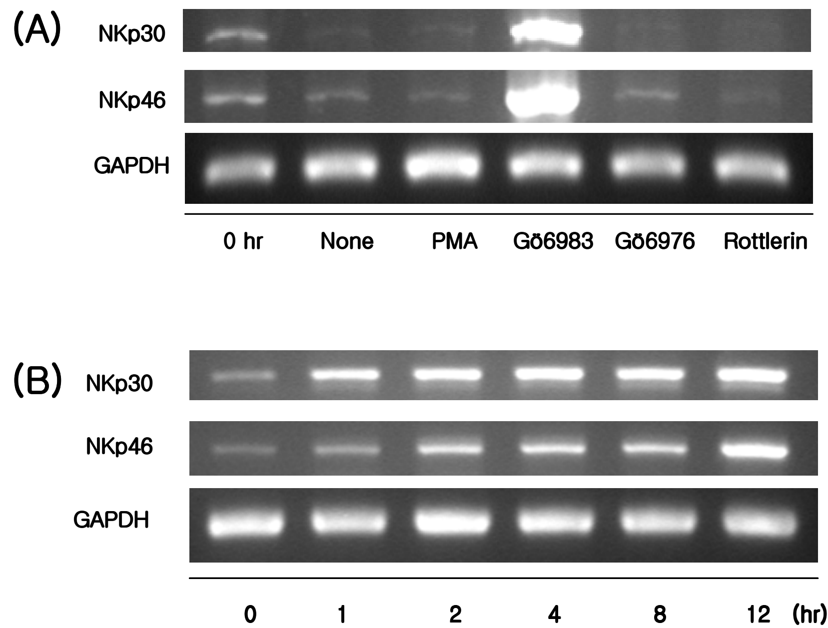


Fig. 10 Effects of PKC inhibitors upon NCR mRNA expression in primary NK cells The NCR mRNA expressions of (A) 12 hr cultured primary NK cells with PMA and indicated inhibitors and (B) Gö6983 treated primary NK cells for indicated time were determined by RT-PCR. The internal control was set by GAPDH.

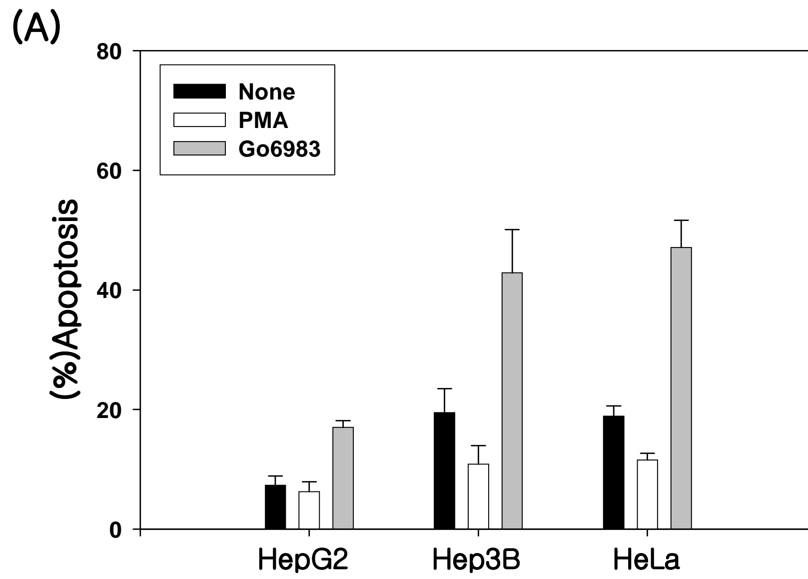
untreated NK cells. Gö6983 also increased expression of NKp46 four-fold (Fig. 9B). Even though the relative MFI ratio of NKp46 increased only about four-fold after Gö6983 treatment, the mean value is quite high considering that substantial amount of NKp46 is constitutively expressed on untreated cultured NK cells (Fig. 9B).

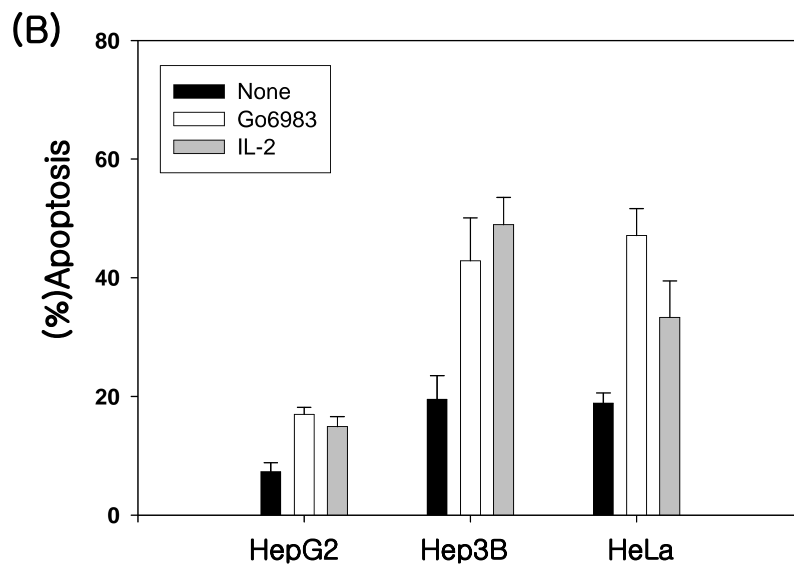
Next, we measured NCR mRNA expression in primary NK cells after Gö6983 treatment. As shown in Fig. 10A, only Gö6983 induced prominent expression of

NKp30 and NKp46 mRNA transcripts. Consistent with the surface expression of NCR, mRNA induction gradually increased until 12 hr (Fig. 10B). In addition, the mRNA levels of NKp30 and NKp46 kept increasing until 24 hr after Gö6983 treatment (data not shown).

7. Effects of Gö6983 on NK cytotoxicity against tumor cell lines

Finally, we investigated whether PMA and Gö6983 could influence target cell lysis through regulation of NCR. The JAM test and ^{51}Cr release assay were performed using PMA and Gö6983 treated NK cells. As anticipated, Gö6983 increased NK cell cytotoxicity while PMA slightly decreased it. Although HepG2 targets were resistant to apoptotic cell death induced by untreated NK cells, Gö6983 treated NK cells induced apoptosis in HepG2. In particular, Gö6983 treated NK cells caused a significant amount of apoptosis on Hep3B and HeLa cells, while PMA reduced NK activity against the target cells (Fig. 11A). In JAM tests (Fig. 11B) and ^{51}Cr release assay (Fig. 11C), Gö6983 appeared to enhance NK cytotoxicity in all target cell lines to a similar degree as IL-2. When NK cells granule release was blocked by mild fixation, apoptotic target cell death measured by JAM test (Fig. 12A) and necrotic


(A)
80
60
40
20
0
(%)Apoptosis
None
PMA
Go6983
HepG2
Hep3B
HeLa


(B)
80
60
40
20
0
(%)Apoptosis
None
Go6983
IL-2
HepG2
Hep3B
HeLa

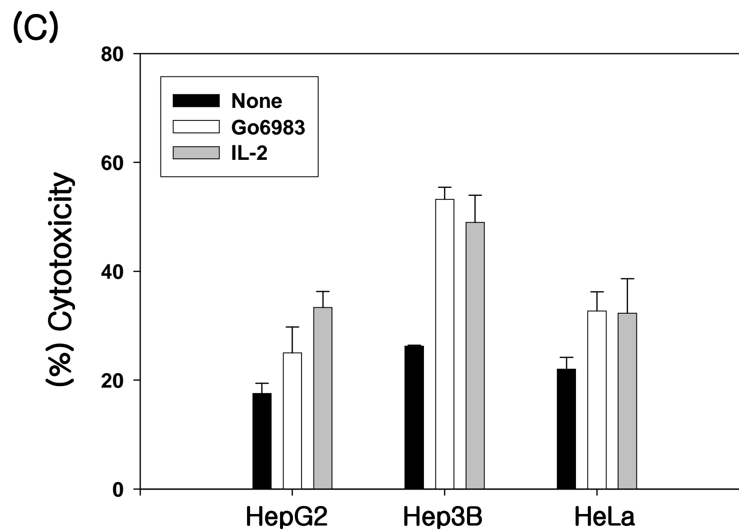


Fig. 11 Effects of a PKC inhibitor (Gö6983) upon NK cytotoxicity The NK activity of target cell lysis were determined by (A, B) JAM test and (C) ^{51}Cr release assay. Primary NK cells were incubated with indicated materials for 24 hr and added to target cells at an E:T ratio of 3:1. NK cells and target cells were cocultured for 2 hr (JAM test) or 4 hr (^{51}Cr release assay). The data are presented as a mean of at least three independent experiments (mean±SD) using NK cells from one donor.

target cell death measured by ^{51}Cr release (Fig. 12B) were not affected by either PMA or Gö6983 treatment. This suggests that Gö6983 and PMA modulate NK cytotoxicity by regulation of cytotoxic granule release.

In summary, Gö6983 treatment of primary NK cells augmented NCR expression and resulted in an increase of NK cytotoxicity against cancer cells, apparently as a consequence of increased granule release.

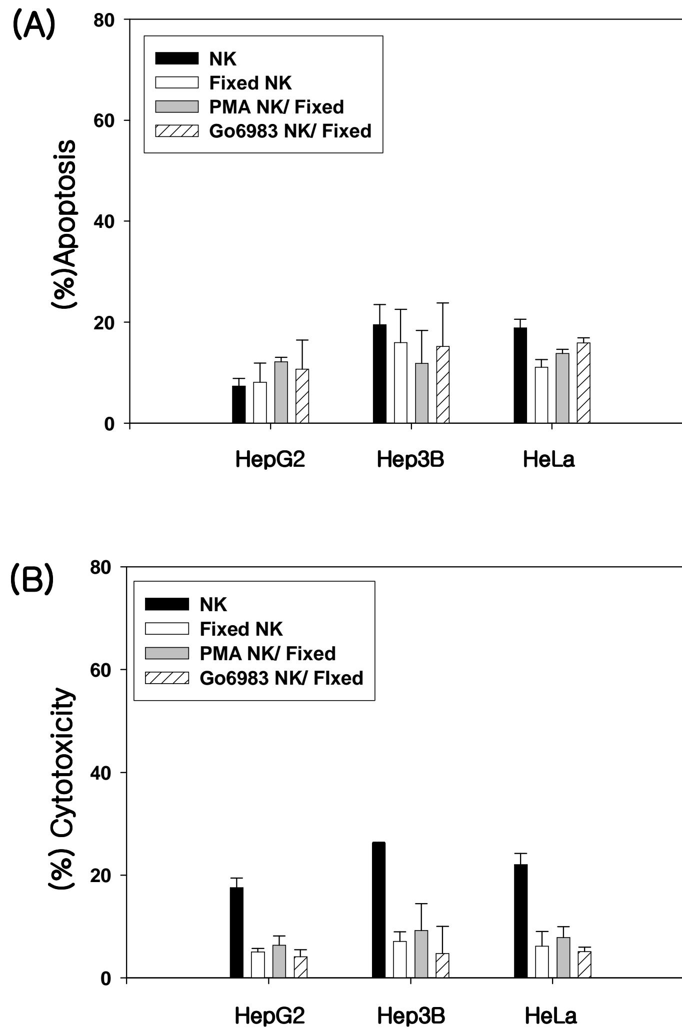


Fig. 12 Cytotoxicity of fixed NK cells against target cells The fixed NK activity of target cell lysis were determined by (A) JAM test and (B) ^{51}Cr release assay. Primary NK cells were incubated with indicated materials for 24 hr and mildly fixed by 0.5% paraformaldehyde for 20 min and then added to target cells at an E:T ratio of 3:1. NK cells and target cells were cocultured for 2 hr (JAM test) or 4 hr (^{51}Cr release assay). The data are presented as a mean of at least three independent experiments (mean±SD) using NK cells from one donor.

IV. DISCUSSION

NK cells express two types of receptors that finely regulate their activity and are characterized by opposite functions. Signaling of inhibitory receptors, one of the two distinct families, is mediated by Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIM) present in their cytoplasmic tail[13]. Some of these receptors recognize MHC class I molecules and they are expressed by all NK cells in a clonally distributed fashion; their interaction with different groups of HLA class I alleles protects normal cells from NK lysis[3, 15, 38]. The other type is activating receptors which usually associate with small transmembrane-anchored adaptor proteins that possess Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) in their cytoplasmic domains[39]. Among the NK activating receptors, three novel NK-cell-specific triggering surface molecules (NKp46, NKp44 and NKp30) have been conspicuous for some time[21-24, 40], although a possible co-receptor role has been proposed for CD2 and the activating forms of the HLA-C-specific receptor (p50) and the CD94-NKG2C heterodimer play a role the NK cytotoxicity[41-44]. Subsequent studies have shown that NKp46, NKp44 and NKp30 appear to play a critical role in the induction of NK-cell-

mediated cytotoxicity and they are now called natural cytotoxicity receptors (NCRs)[18].

NCR expression is directly correlated with NK cytolytic activity, and low NCR surface density and NK cytotoxicity have been implicated in some diseases, including HIV-1 infection[33], *M. tuberculosis* infection[45], myeloid leukemia[46] and some other tumors. However, the regulatory mechanism of NCR expression on NK cells are not yet well understood. Therefore, this study attempted to determine the factors affecting NCR surface expression on NK cells. We first constructed an artificial NCR expression system for this purpose (Fig. 1). Since NCRs are almost exclusively expressed on NK cells[18], a similar cell line which does not express NCRs was required, and Jurkat was used for transfection experiments. Jurkat expresses the CD3ζ chain which is an adaptor protein for NKp46 and NKp30, but does not express DAP12 for NKp44[47]. Thus, we transfected NKp30 and NKp46 expression vectors to Jurkat cell lines. PMA was used to stimulate NCR transfectants, and the surface expression of wild type NKp30 and NKp46 were observed as a function of time using flow cytometry (Fig. 2). The regular increase and decrease of NCR expression was also monitored by immunoblotting (Fig. 4). These results clearly demonstrate that

total protein and surface expression levels of NKp30 and NKp46 were altered after PMA stimulation.

While diacylglycerol, the physiologic activator of protein kinase C, is present only transiently in cells, PMA degrades very little and remains for comparatively longer periods[48]. Therefore, over time, protein kinase C presumably acts in both positive and negative ways depending on the function of its target proteins[49]. In early cellular responses protein kinase C appears to act synergistically, but sometimes there is a negative feedback control, such as downregulation of some receptors[50, 51]. Thus, it is quite possible that NCR expression on Jurkat T cell lines were influenced by continuous PMA stimulation. However, NCR transcript expression in stable cell lines did not show any significant change. This suggests that NCR expression may be regulated at a post-transcriptional level.

We predicted that PKC activation by PMA would cause alteration in protein expression level and the conformation of PKC binding sites that might result in post-transcriptional regulation of NCR expression in Jurkat T cells. To investigate which regions of NCRs are critical for this phenomenon, we made a series of point mutants

in NKp30 and NKp46. In particular, we substituted alanine for serine and threonine residues in the cytoplasmic tail region which were expected to be PKC binding sites (Fig. 5). Interestingly, most of the NCR mutants behaved similarly to the wild type. However, the expression pattern of the NKp46 S288A clone was very different. Unlike the other mutant proteins, NKp46 S288A surface expression was almost undetectable. Furthermore, NKp46 S288A expression was not affected by PMA treatment. These results suggest that Ser288 of NKp46 might play an important role in NKp46 expression and its regulation by PMA (Fig. 5C).

We next investigated whether NCR expression is similarly regulated by PMA in NK cells. In fact, NKp30 expression was not significantly changed after PMA stimulation (Fig. 6), and NKp46 expression slightly decreased (Fig. 7B, D). Compared to the Jurkat transfectants, a different response to PMA stimulation seems to occur in primary NK cells. Actually, an acute inhibitory effect has been observed in primary NK cells after brief pretreatment with PMA[52]. Furthermore, NCR mRNA expression after PMA stimulation paralleled the surface expression (Fig. 7E). These results indicate that NCR expression is differently regulated in NK cells compared

with the artificial overexpression system of Jurkat T cells. Therefore, we screened other candidate molecules which are expected to act as regulators of NCR expression in NK cells. First of all, cytokines such as IL-2, IL-8, IL-12, IL-15, IL-18, IFN-α1 and IFN-α2b, which are known as NK activators or immune system stimulator[53-56], did not appear to induce any notable change in NCR expression on primary NK cells (Fig. 6).

According to previous reports, more than 90% of cytosolic PKC is translocated to the membrane in NK cells exposed to PMA, and translocation is followed by the appearance of PKM. PKC is also activated by calpain proteolysis and this proteolytic activation produces PKM[49]. Based on this information, we investigated the effects of inhibitors which are related to cytoplasmic signal transduction on NCR expression in primary NK cells (Fig. 8). PD98059 blocks ERK1/2 activation and inhibits cytolytic activity of NK cells against some targets[57], PD150606 is a calpain inhibitor, and lactacystin is a proteosome inhibitor[58]. These inhibitors did not affect NCR expression (Fig. 8), although they are known to influence NK cytolysis[49].

Since PMA, a PKC activator, appeared to affect the NCR expression in primary NK cells (Fig. 7), we focused on how PMA and PKC inhibition affect NCR

expression. We selected typical inhibitors of PKC isotypes: Rottlerin (novel PKC), Gö6976 (conventional PKC), Gö6983 (broad PKC isotypes) and BisIII (PKC/PKA)[59]. Of these inhibitors, Gö6983 induced an astonishing increase of NCR expression on primary NK cells. NKp30 expression increased to a greater extent than NKp46 (Fig. 9). Interestingly, NCR transcript expression in primary NK cells paralleled the surface expression patterns (Fig. 10). This suggests that Gö6983 treatment regulates NCR expression in NK cells at the transcriptional level.

Finally we investigated whether PMA and Gö6983 influence target cell lysis by NK cells through the regulation of NCR expression. As expected, Gö6983 treatment of primary NK cells induced increased NCR expression on NK cells, and their cytolytic activity also increased (Fig. 11). JAM test and ^{51}Cr release assay data demonstrated that Gö6983 significantly increased NK cell-induced apoptosis in Hep3B and HeLa (Fig. 11A), and necrosis in all target cells (Fig. 11C). However, PMA appeared to slightly decrease the cytolytic activity of NK cells. Furthermore, JAM test and ^{51}Cr release assay of mildly fixed NK cells showed that the increase of NK cytotoxicity influenced by NCR expression was induced via the granule release

pathway (Fig. 12A, B).

The PKC isotypes inhibited by Gö6983 are PKC α, β, γ, δ and PKC ζ. PKC α, β, γ and δ are also inhibited by Gö6976 and rottlerin. Thus, PKC ζ, is the only isotype inhibited by Gö6983 alone. This suggests that PKC ζ might be involved in the upregulation of NCR expression upon Gö6983 treatment, but this has not been definitively demonstrated in this study. Although more elaborate studies are needed to elucidate the molecular mechanism of NCR upregulation by Gö6983, our data demonstrate that Gö6983 could be used as a NK activating molecule. Most importantly, NCR up-regulation by Gö6983 is directly associated with an increase of NK cytotoxicity against cancer cells.

V. CONCLUSION

1. Expressions of NKp30 and NKp46 in Jurkat T cells appeared to increase by PMA treatment until 8 hr, but gradually decreased after that time to lower levels than initial state.
2. NKp30 and NKp46 mRNA expressions were not significantly changed after PMA stimulation in Jurkat transfectants.
3. NKp30 and NKp46 total protein expressions after PMA stimulation increased until 6~8 hr and then gradually decreased in Jurkat T cells.
4. Mutation studies revealed that Ser288 of NKp46 might play an important role in NKp46 expression and in the regulation by PMA.
5. In primary NK cells, most of cytokines such as IL-2, IL-8, IL-12, IL-15, IL-18, IFN-α1 and IFN-α2b did not appear to significantly alter the NCR expressions.
6. Inhibitors of MAP kinase, calpain and proteosome also did not induce any notable change of NCR expression in primary NK cells.
7. PMA slightly down-regulated surface and mRNA expression of NKp46 on

primary NK cells.

8. Although other PKC inhibitors did not induce or suppress the NCR expression, Gö6983 induced remarkable increment of NCR expression on NK cells.

9. Up-regulation of NCR on NK cell surface by Gö6983 caused the increase of NK cytotoxicity against hepatocellular carcinoma cell lines and HeLa.

10. The enhancement of NK cytotoxicity by the increase of NCR expressions was attributed to the cytotoxic granule release pathway of NK cytotoxicity.

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Abstract (in Korean)

자연살 세포(NK 세포)에서의 자연 세포독성 수용체(NCR)의 발현 조절

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자연살 세포(Natural Killer cells; NK 세포)는 종양세포 및 바이러스에 감염된 숙주세포를 제거하는 기능을 수행하며 부적절한 골수 이식을 거부하고 수상돌기세포를 통해서 T 세포의 면역반응을 조절하는 등 생체 내 면역계의 방어 기작의 제 1선에서 작용하는 매우 중요한 행동세포이다. 자연살 세포는 표면에 활성화 수용체(activating receptor)와 억제 수용체(inhibitory receptor)로 구분되는 여러 수용체들을 발현하고 있는데, 활성화 수용체 중에서도 자연 세포독성 수용체(Natural Cytotoxicity Receptors; NCRs)가 자연살 세포의 세포독성을 매개하는 주된 수용체이다. NKp46 (NCR1), NKp44 (NCR2) 그리고

NKp30 (NCR3)으로 구성되는 이 자연 세포독성 수용체들은 자연살 세포에서만 발현되고 그 표면 밀집도는 자연살 세포의 세포독성과 직접적으로 연관된다.

본 연구는 자연살 세포에서의 자연 세포독성 수용체의 발현에 영향을 미치는 요인을 찾고 그 요소들이 어떻게 수용체의 발현을 조절하는지 알아보고자 하였다. Jurkat T 세포주를 사용하여 자연 세포독성 수용체를 발현하는 세포주를 구축하여 PMA 자극에 의해 변화하는 수용체의 발현을 유세포 분석과 RT-PCR과 immunoblotting으로 확인하였다. PMA에 의해서 NKp30과 NKp46의 발현이 8시간 정도까지 증가하다가 점차적으로 줄어들었으며 mRNA 양에서는 별 다른 변화를 관찰 할 수 없었다. 그에 비해 이들 자연 세포독성 수용체의 총 단백질량은 표면 발현과 비슷한 경향을 보였고 이러한 현상에 NKp46의 288번 serine이 중요한 역할을 하고 있음을 밝혀내었다.

실제 자연살 세포에서의 자연 세포독성 수용체 발현에 영향을 미치는 인자들을 찾아내기 위해 기존에 면역계를 활성화 시킨다고 알려져 있던 여러 cytokine들을 탐색해 보았으나 크게 영향을 미치는 요소를 찾지 못했고, MEK 억제제인 PD98059, calpain 억제제인 PD150606과 proteosome 억제제인 Lactacystin도 자연 세포독성 수용체의 발현에 별 영향을 미치지 않는 것으로

나타났다. PMA 자극에 의해서 실제 자연살 세포에서는 NKp46의 발현이 다소 줄어들었다. PMA가 대표적인 PKC 활성제이므로 PKC 억제제가 자연 세포독성 수용체의 발현에 미치는 영향을 살펴보았다. 대부분의 PKC 억제제가 수용체의 발현에 아무런 영향을 미치지 못했는데, PKC α, β, γ, δ와 ζ를 억제하는 Gö6983에 의해서 NKp30과 NKp46의 발현이 현저히 증가하였다. Gö6983은 NKp30과 NKp46의 발현을 transcriptional level에서 조절함을 확인하였고 이렇게 증가된 수용체의 발현이 HepG2, Hep3B와 HeLa 암 세포주에 대한 자연살 세포의 세포독성의 증가에까지 영향을 미치는 것을 알 수 있었다.

결론적으로, PMA에 의해서 다소 감소하고 Gö6983에 의해서 크게 증가하는 자연 세포독성 수용체의 발현이 자연살 세포의 세포독성을 조절하고, 따라서 이들 약물은 생체 내 면역계 활성에도 영향을 미칠 수 있을 것이라 사료된다.

핵심 되는 말: 자연살 세포, 자연 세포독성 수용체, PKC, PMA, Gö6983