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Molecules and Cells



# IL-15-Induced IL-10 Increases the Cytolytic Activity of Human Natural Killer Cells

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**Interleukin 10 (IL-10) is a multifunctional cytokine that regulates diverse functions of immune cells. Natural killer (NK) cells express the IL-10 and IL-10 receptor, but little is known about the function of IL-10 on NK cell activation. In this study, we show the expression and role of IL-10 in human NK cells. Among the cytokines tested, IL-15 was the most potent inducer of IL-10, with a maximal peak expression at 5 h after treatment. Furthermore, IL-10 receptor was shown to be expressed in NK cells. IL-10 alone had a significant effect on NK cytotoxicity which additively increased NK cell cytotoxicity in the presence of IL-15. Neutralizing IL-10 with anti-IL-10 antibody suppressed the inductive effect of IL-10 on NK cell cytotoxicity; however, IL-10 had no effect on IFN-γ or TNF-α production or NK cell activatory receptor expression. STAT signals are implicated as a key mediator of IL-10/IL-15 cytotoxicity response. Thus, the effect of IL-10 on NK cells is particularly interesting with regard to the STAT3 signal that was enhanced by IL-10 or IL-15.**

## INTRODUCTION

The ability of natural killer cells (NK) to mediate the primary immune response against viruses, intracellular bacteria, tumors and parasite-infected cells has led to their association with other lymphocyte activity modulation of the adaptive and innate immune defense system (Lanier and Phillips, 1992; Ortaldo and Longo, 1988; Spits et al., 1995). NK cells also regulate other aspects of the immune response with inflammatory cytokine production. In particular, the ability to produce IFN-γ and TNF-α and to lyse infected cells is important in the body's defense against infection (Scharton-Kersten and Sher, 1997). In addition, studies have demonstrated that IFN-γ or TNF-α secretion can promote distinct types of immune response controlled by type 1 (Th1) and type 2 (Th2) subpopulations of T cells (Scharton and Scott, 1993).

Like Th1 and Th2 cells, human NK cells have a distinct pattern of cytokine secretion. The ability of IL-15 to directly stimulate NK cell secretion of IFN-γ is at the center of these responses. Other factors such as TNF-α, IL-1, IL-2, IL-15, and CD28, serve as costimulatory factors, enhancing IFN-γ production by NK cells, as well as NK cell proliferation and cytotoxicity (Hunter et al., 1997a; 1997b; 1997c; Nandi et al., 1994; Tripp et al., 1993). The *in vivo* existence of human NK cell subsets similar to Th1 and Th2 cells was demonstrated in the production of TGF-β and IL-10; therefore, the regulatory function of NK cells still needs elucidation (Asseman et al., 1999; Fiorentino et al., 1989).

The well-known NK cell activatory cytokine, IL-15, is a member of the γ-chain receptor family of cytokines, which also includes IL-2, IL-4, IL-7, IL-9 and IL-21. IL-15 coordinates the response of NK innate and adaptive immune cells for host protection (Kennedy et al., 2000; Ma et al., 2006). Treatment with IL-15 or IL-2 results in a similar receptor signal chain reaction and an IL-15 response followed by long-lasting responsiveness to IL-2 and trans-presented IL-15 (Becknell and Caligiuri, 2005; Waldmann, 2006). This sequence of events does not depend on initial stimulus, even though IL-15 is more efficient at initiating early activation events than IL-2 (Dubois et al., 2008; Sun et al., 2003). IL-15 stimulation of NK cells is reasonable for monitoring the rapid primary immune response.

IL-10 has important immunoregulatory functions and, a wide spectrum of biological activity. IL-10 produced by macrophage, type 1 helper T cells and some NK cells has been reported to vary in function based on the tumor environment (Moore et al., 1993). IL-10 was initially described as a cytokine-synthesis inhibitory factor (CSIF) due to its downregulatory effects on cytokine production from Th2 cells. Further investigations revealed that IL-10 inhibits cytokine production from T cells by downregulating antigen -presentation capacities of macrophages and monocytes (Murray et al., 1997). Furthermore, the role of IL-10 in the generation and maintenance of NK and regulatory T cells has been actively investigated. In particular, the broad ability of IL-10 to inhibit the inflammatory cytokine secretion and negative effects to T lymphocytes suggests that IL-10 acts as an antagonist of NK cell activation (D'Andrea et al., 1993; Neyer et al., 1997; Tripp et al., 1993); however, it is unknown whether IL-15-induced IL-10 by NK cells enhances cell activation by cooperating with other stimulatory cytokines or negatively regulates the inflammatory system. In this study, we expressed IL-10 and its receptor in human NK cells and impli-

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cated IL-10 as a positive regulator of NK cell cytotoxicity.

## MATERIALS AND METHODS

### Cell culture and reagents

The human NK cell line NK92 cells (American Type Culture Collection, ATCC) were cultured in alpha MEM (Gibco), supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Thermo Scientific HyClone) and IL-2 (10 ng/ml). NK92 cells cultured with IL-2 (10 ng/ml) were deprived in medium alone for 24 h and re-stimulated with IL-15.

Umbilical cord blood (UCB) was collected from umbilical veins after neonatal delivery, with informed consent and following the guidance of the local institutional review board (IRB). To prepare hematopoietic stem cell (HSC)-derived mature NK cells (mNK cells), $CD34^+$ HSCs were isolated from UCB using the CD34 MicroBead Kit (Miltenyi). $CD34^+$ HSCs were differentiated into NK cell precursors by incubating the cells in Myelocult H5100 supplemented with SCF (30 ng/ml) and Flt3 ligand (50 ng/ml) for 14 days. NK cell precursors were differentiated into mNK cells by IL-15 (30 ng/ml) stimulation for an additional 14 days. mNK cells (> 97% $CD56^+CD3^-$ cells) were maintained in Myelocult H5100 with IL-15 (10 ng/ml) and used for the functional assays. Recombinant human SCF, Flt3 ligand, IL-10, IL-12 and IL-15 were purchased from PeproTech. Naïve NK cell was isolated from blood of a healthy person using a NK cell isolation kit (MACS, Miltenyi biotec) and prepared for the experiment.

### IFN-γ, TNF-α and IL-10 measurement

The culture supernatants were collected and assayed by a commercial enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences) following the manufacturer's recommendation. The total concentrations of IFN-γ, TNF-α and IL-10 of $10^6$ cells per well of culture supernatants were determined by ELISA.

### NK cell cytotoxicity assays

Cytotoxicity was examined using a standard 4 h $^{51}Cr$-release assay. $^{51}Cr$-labeled target K562 cells ($10^5$ cells/well) and serial dilutions of NK cells were plated in triplicate. The $^{51}Cr$ released into the supernatant was measured using a γ-counter. The percentage of specific lysis was calculated using the following formula: (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100.

### Real-time PCR

Total RNA was extracted using TRIZOL (Invitrogen) and reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega) with random primers (Takara Bio). Real-time PCR was performed using a Dice TP 800 Thermal Cycler and the SYBR Premix Ex Tag (Takara Bio). The data were normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript. The primer sequences were as follows: 5′-GTC CAA CGC AAA GCA ATA CA 3′ and 5′ CTC TTC GAC CTC GAA ACA GC 3′ for IFN-γ, 5′ TGC CTT CAG TCA AGT GAA GAC 3′ and 5′ AAA CTC ATT CAT GGC CTT GTA 3′ for IL-10, 5′ CAG CCT CAA GAT CAT CAG CA 3′ and 5′ GTC TTC TGG GTG GCA GTG AT 3′ for GAPDH.

### Western blotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% SDS, 1% NP-40, and 1 mM EDTA), supplemented with PBS containing 1% Tween-20 and complete proteinase inhibitor (Roche) and Phosphostop (Roche). The cell lysates were resolved by SDS-PAGE using 8 or 12% gels and transferred to a PVDF membrane (EMD Millipore). The membrane was probed with antibodies specific to the following proteins: p-STAT/STAT 1, 3, 4 and 5 (Cell Signaling) and GAPDH (Assay Designs). After incubation with peroxidase-conjugated anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch), the signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce) or Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore).

### NK cell neutralization

NK92 cells were cultured in a 12-well plate ($10^6$ cells/well), deprived for 24 h and incubated for 12 h with anti-IL-10 antibody, purified NA/LE rat anti-human IL-10 10 μg/ml (BD Biosciences), control antibody rat IgG2κ 10 μg/ml (BD Biosciences), anti-IL-10R antibody and anti-CD210/IL-10R 10 μg/ml (Biolegend) neutralization antibody before the experiment.

### Flow cytometry

PE-anti-human CDw210 antibody (BD Biosciences) was used for flow cytometry. NK cells ($10^4$) were stained with 1 μl of the indicated antibodies in a PBS staining buffer containing 1% FBS and 0.01% $NaN_3$, for 30 min at 4°C and washed for flow cytometry. NK receptors were stained with NKG2D, NKp30 NKG2D and 2B4(CD244) antibody conjugated with PE (BD Biosciences) for the histogram analysis.

## RESULTS

### IL-15-induced IL-10 expression in NK cells

The activation of NK cells by IL-15 is accompanied by an increase in proliferation and cytolytic effect. Exposing NK92 cells to IL-15 (30 ng/ml) enhanced IFN-γ mRNA expression with a maximal peak at 5 h (Fig. 1A). Simultaneously, IL-15 increased IL-10 mRNA expression with almost the same kinetics (Fig. 1B). Protein analysis showed that IFN-γ (Fig. 1C) and IL-10 expression (Fig. 1D) were almost the same as mRNA expression. ELISA data confirmed that IFN-γ (Fig. 1E) and IL-10 production (Fig. 1F) were induced by IL-15. At this condition, IL-15 markedly enhanced NK cell specific killing activity against $^{51}Cr$-labeled K562 cells (Fig. 1G). The kinetics of IL-15-induced IL-10 mRNA expression were similar in mNK cells as in NK92 cells (Fig. 1H). Next, we compared IL-10 production induced by several known NK cell activators such as IL-2 (10 ng/ml), IL-12 (30 ng/ml), IL-18 (50 ng/ml), and IL-21 (30 ng/ml). The most potent inducer was IL-15 (Fig. 2A), which accumulated IL-10 secretion in a time-dependent manner (Fig. 2B) with an optimal dosage of 30 ng/ml (Fig. 2C). Collectively, IL-10 was up-regulated during the IL-15-induced activation of NK cells.

### IL-10R expression and signaling in NK cells

To understand the functions of NK cell-derived IL-10 in NK cell activation, first we evaluated expression levels of IL-10R in NK92 and mNK cells. The FACS revealed that IL-10R expression was readily detected in NK cells (Figs. 3A and 3B). The relative IL-10R level was more abundant in mNK cells than in NK92 cells. Studies have shown that T-cell activation possibly leads to down-regulation of IL-10R transcript (Nagalakshmi et al., 2004); however, there was no change of IL-10R mRNA expression in NK cells in response to IL-15 (data not shown). To assess for functional IL-10R in NK cells, we analyzed STAT phosphorylation, a known IL-10R-mediated signaling event. We observed that IL-10 activates the tyrosine phosphorylation of STAT1, 3 and 4 through IL-10R (Fig. 3C). IL-10 was expected to affect NK cell function by binding to IL-10R and phosphory-

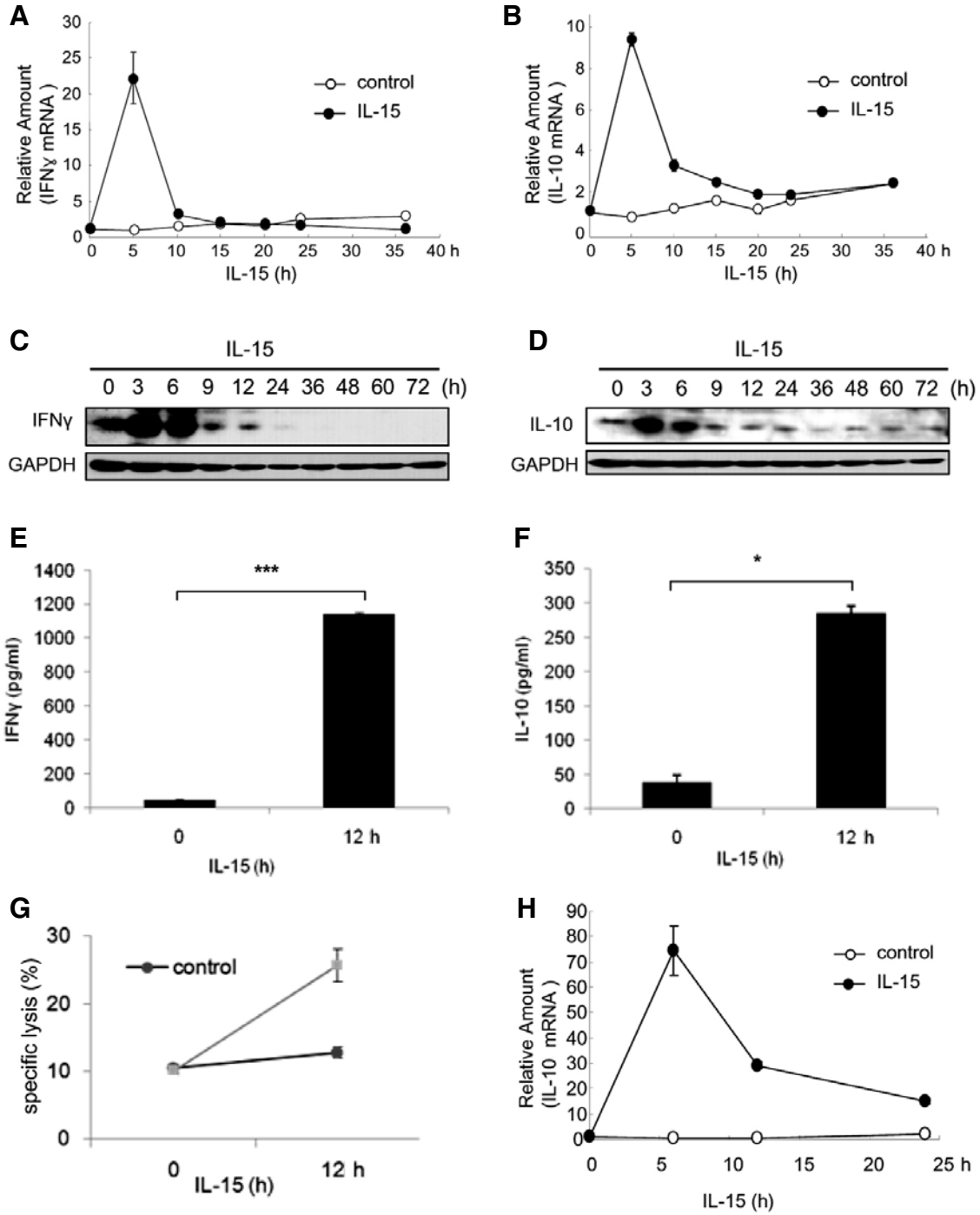


**Fig. 1.** IL-15 increases IL-10 expression in human NK cells. Pre-deprived NK92 cells were activated with IL-15 (30 ng/ml) for various times and mRNA expression level was measured by qRT-PCR. (A, B) Data representation of the IFN-γ and IL-10 mRNA ratio relative to the amount of mRNA loaded. (C, D) Extract of IL-15-treated NK92 cell was separated by SDS-polyacrylamide gel electrophoresis and analyzed for IFN-γ and IL-10 expression by Western blot. (E, F) The amounts of secreted IFN-γ and IL-10 were determined by ELISA. (G) NK cytolytic activity after IL-15 treatment was tested by $^{51}$Cr-release assay against K562 cells. (H) IL-15-induced IL-10 mRNA expression was similar to mNK (H). Cytolytic assay, mRNA qRT-PCR and ELISA results represent three independent experiments and the mean values ± S.E.M. of duplicate wells. P values, paired two-tailed *t*-test. *p < 0.05; **p < 0.01; ***p < 0.001.

lating specific STATs.

**The effects of IL-10 on NK cell activity**

We tested whether combining IL-15 and IL-10 synergistically enhances cytokine production. Results show that there was no change in the production of IFN-γ (Fig. 4A) and TNF-α (Fig. 4B) by combining IL-10 and IL-15. Cytokine production may have a different regulatory pathway than IL-10-enhanced NK cell acti-

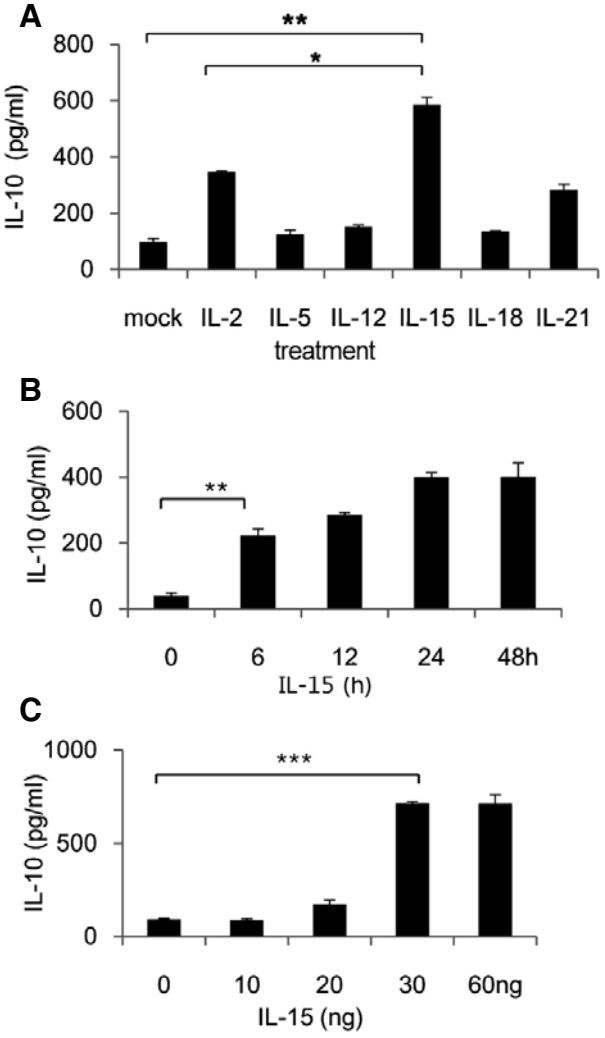


**Fig. 2.** IL-15 dominantly upregulates IL-10 expression in NK cells. (A) NK92 cells were cultured with different cytokines, including IL-2 (10 ng/ml), IL-5 (30 ng/ml), IL-12 (30 ng/ml), IL-15 (30 ng/ml), IL-18 (50 ng/ml) and IL-21 (30 ng/ml) for 12 h. (B, C) NK92 cells were stimulated once with IL-15 (30 ng/ml) for various times or with increasing concentrations of IL-15 for 24 h. Secreting IL-10 was quantified by ELISA. Results represent two independent experiments and the mean values ± S.E.M. of duplicate wells. P values, paired two-tailed *t*-test. *p < 0.05; **p < 0.01; ***p < 0.001.

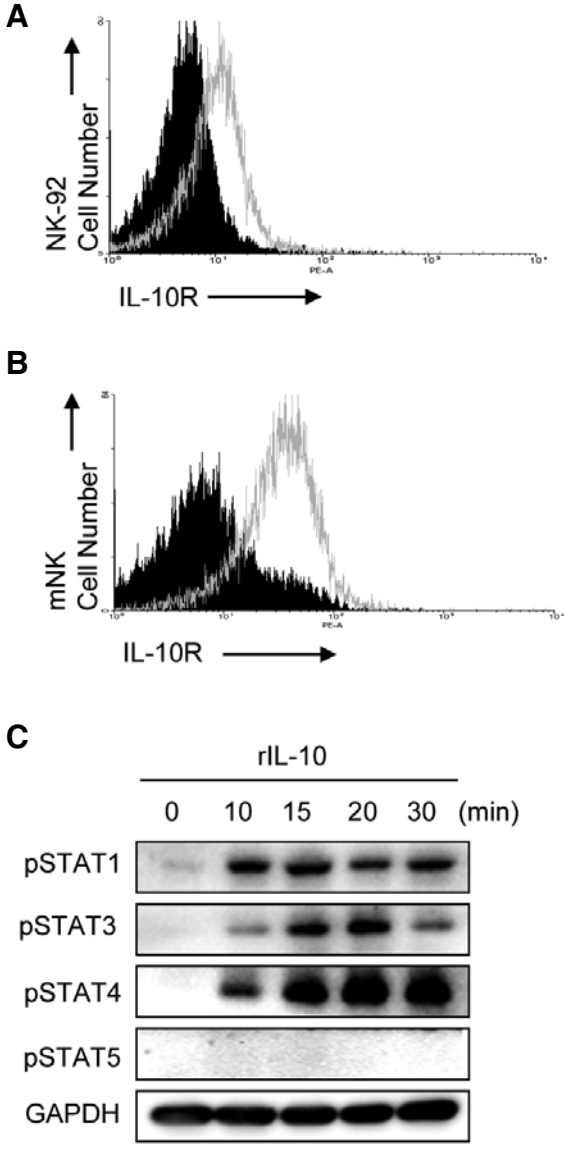


**Fig. 3.** Expression of IL-10R and STAT signal activation in NK cells. (A, B) Expression of IL-10R on NK92 and mNK was analyzed by FACS using IL-10R-PE antibody (CDw210, which recognizes IL-10Rα and IL-10Rβ). The gray line indicates IL-10R expression. (C) NK92 cells stimulated with IL-10 (30 ng/ml) for 15 min show specific STAT phosphorylation. Results are expressed from triplicate samples (A) as mean values ± S.E.M. Data are representative of two (A, B) or three (C) independent experiments.

vation.

On the basis of previous studies and our present observation, we assessed the effects of IL-10 and IL-15 derived STAT on NK92 and naïve NK cell cytotoxicity. Our data showed that IL-10 alone elevated NK cell cytotoxicity activity (Figs. 4C and 4D); however, the combination of IL-10 (30 ng/ml) with a suboptimal concentration of IL-15 (30 ng/ml) had an additive effect on NK (Figs. 4C and 4D) cell cytotoxicity. In addition, IL-10 neutralization with anti-IL-10 mAb was performed. Anti-IL-10 mAb reduced NK cell cytolytic activity enhanced by combining IL-10 and IL-10/IL-15; however, anti-IL-10 antibody had no effect on IL-15-mediated cytotoxicity (Figs. 4C and 4D), meaning that IL-15 induced IL-10 did not affect NK cytotoxicity. The secreted concentration of IL-10 induced by IL-15 was less than 1 ng/ml (Fig. 2), and the external recombinant IL-10 was 30 ng/ml. Therefore, the concentration of secreted IL-10 by IL-15 might be not sufficient to regulate cytotoxicity. Thus, IL-10 and IL-10R-mediated STAT signaling represents an additive effect of autocrine cytokines.

**The effects of IL-10 on signaling pathways in activated NK cells**

The interaction of IL-10 and IL-10R engages the activation of the Jak family tyrosine kinases Jak1 and Tyk2, which are constitutively associated with IL-10R1 and IL-10R2. IL-10 induces tyrosine phosphorylation and activation of the latent transcrip-

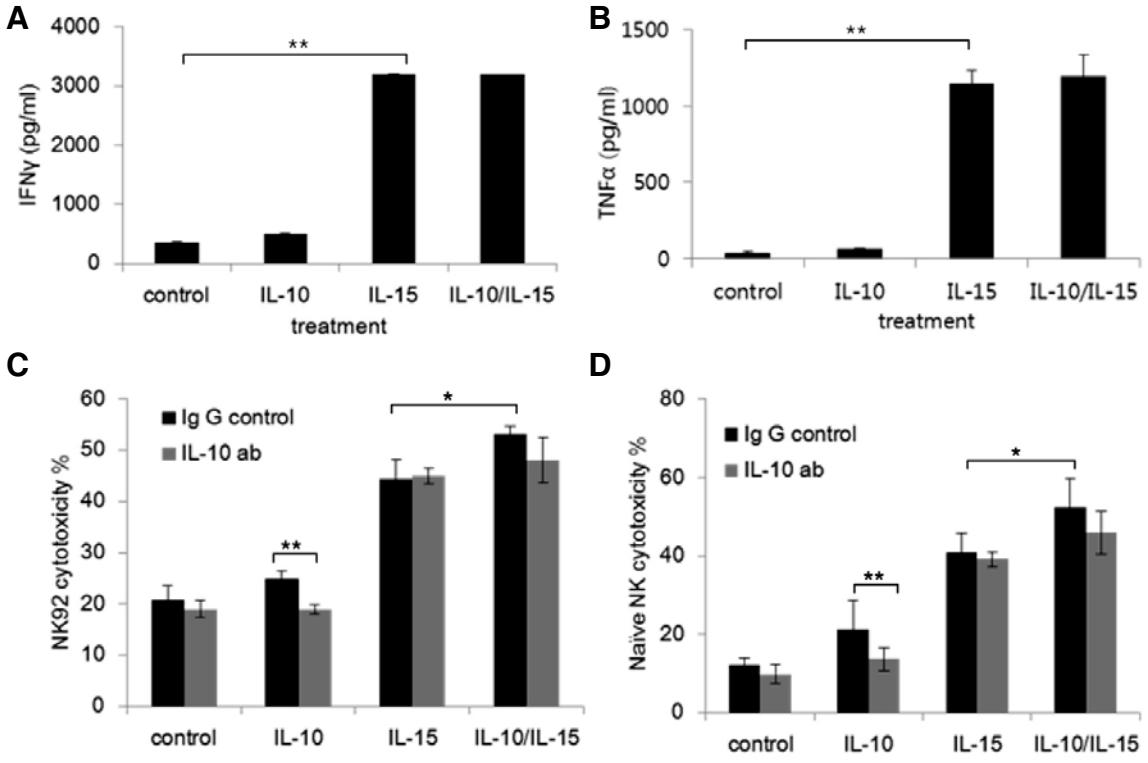


**Fig. 4.** Effects of IL-10 on IL-15 induced NK92 cytotoxicity and cytokine production. (A, B) Cells were stimulated with IL-10 or/and IL-15 for an ELISA. The secreted level of IFN-γ and TNF-α in the culture supernatants was measured as described in "Materials and Methods". (C, D) NK92 and naïve NK cells stimulated with IL-15 (30 ng/ml) for 12 h with or without IL-10 (30 ng/ml) show specific killing activity against $^{51}$Cr-labeled K562 cells. NK cells cultured with neutralizing anti-IL-10 mAb (10 μg/ml) or control IgG (10 μg/ml) for 12 h were stimulated with IL-10 and/or IL-15. Relative NK92 cytolytic effects of $^{51}$Cr-labeled K562 cells were counted. Results are expressed from duplicate (C, D) or triplicate (A, B) samples as mean values ± S.E.M. Data are representative of two (C, D) or three (A, B) independent experiments. P values, paired two-tailed *t*-test. *p < 0.05; **p < 0.01; ***p < 0.001.

tion factors STAT1, STAT3 and STAT4 (Weber-Nordt et al., 1996). To understand the molecular mechanism of additive effects of IL-10 and IL-15 on NK cell cytotoxicity, we monitored the activation markers STAT, Erk, and Akt phosphorylation in NK92 cells. IL-15 dramatically induced STAT1, STAT3, STAT4, Akt, and Erk phosphorylation. IL-10 also activated STAT1, STAT3 and Erk, but not STAT5 and Akt (Figs. 5A and 5B). Importantly, there were some additive effects on STAT1 and STAT3 phosphorylation when cells were treated with both cytokines. We next confirmed the specificity of IL-10 to STAT1 and STAT3 phosphorylation by treatment with anti-IL-10R mAb. STAT3 acted as a key mediator of the signaling cascade of IL-10, which is secreted by IL-15; however, it seems that IL-10-mediated STAT3 activation is not directly related to NK activation. More studies are required to dissect the cross-talk between IL-10-activated STAT3 signaling and NK cell activation (Fig. 5A).

The NK cell surface receptors are positively necessary for modulating the killing effect of NK cells. We therefore investigated whether NK cell receptors are modulated by IL-10 treatment by FACS assay. After IL-10 and/or IL-15 incubation for 24 h, IL-15 induced the expression of NK cell receptors in NK92 cells (Fig. 5C). Well-known NK activation receptors, such as NKp30 and NKG2D, were especially induced by IL-15; however, IL-10 had no effect on receptor expression (NKp30, NKp44, NKp46, NKG2D and 2B4) in the same condition. Both cytokines had no effect on NK cell receptor expression in naïve NK (Fig. 5D). Therefore, these results suggest that IL-10 did not change the receptor's expression but affected the cytotoxicity via other mechanisms.

## DISCUSSION

Although previous studies introduce IL-10 as an inhibitory cytokine, we studied the effects of IL-10 on *in vitro* NK cell activity. The molecular mechanisms underlying the postulated immunostimulatory activity of IL-10 have yet to be elucidated. The processes of immunoregulatory roles described in this study are as follows: 1) NK cells induce cytokine IL-10 expression by IL-15; 2) IL-10 additively enhances NK cell cytotoxicity; and 3) the additive effect of IL-10 on IL-15 might be mediated by STAT signaling pathways.

The well-known NK cell activators IL-2 and IL-15 share a common intermediate heterodimer receptor formed by the association of two chains, IL-2/IL-15Rβ (CD122) and the γ-chain (CD132) (Ma et al., 2006). IL-10 secretion divergence and discrepancy effects of IL-2 and IL-15 on NK cells have been shown (Ma et al., 2000; 2006; Waldmann et al., 2001). IL-15 has a more stimulatory tendency and induces a stronger proliferative activity than IL-2 (Sun et al., 2003). Also, the physiological relevance of those cytokine receptors indicates the enhanced NK cell sensitivity to IL-15 in early activation and redirects responses toward IL-2. This might provide the relevance for IL-15 to induce IL-10 on NK cells (Pillet et al., 2009).

Although IL-10 is associated with inhibition of cell mediated immunity, its stimulatory properties have been studied. NK cell

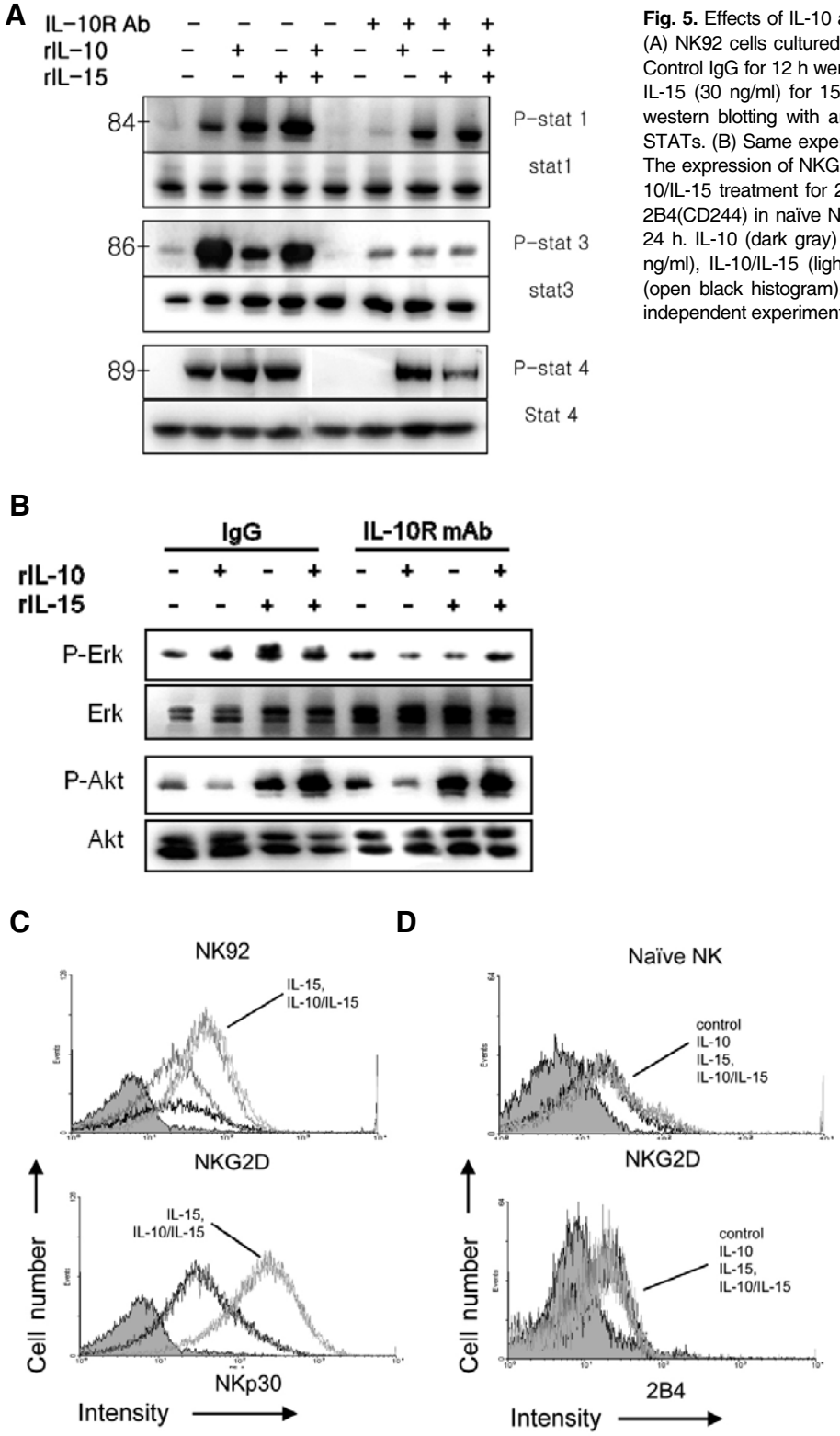


**Fig. 5.** Effects of IL-10 and IL-15 on protein phosphorylation. (A) NK92 cells cultured with neutralizing anti-IL-10R mAb or Control IgG for 12 h were treated with IL-10 (30 ng/ml) and/or IL-15 (30 ng/ml) for 15 min. Cell lysates were analyzed by western blotting with antibodies specific for phosphorylated STATs. (B) Same experiment analyzed for ERK and Akt. (C) The expression of NKG2D and NKp30 in NK92 cells after IL-10/IL-15 treatment for 24 h. (D) The expression of NKG2D, 2B4(CD244) in naïve NK cells after IL-10/IL-15 treatment for 24 h. IL-10 (dark gray) (30 ng/ml), IL-15 (medium gray) (30 ng/ml), IL-10/IL-15 (light gray) (30 ng/ml) each and control (open black histogram). The data are representative of two independent experiments.

cytotoxicity against autologous macrophages was increased in the presence of IL-10 by elevating NK cell activation receptors (Schulz et al., 2010). Chronic infections are characterized by the development of progressive T cell dysfunction, and tolerogenic impact of IL-10 has been illustrated in several studies. Therefore, the progressive immune dysfunction observed in chronic viral infections might be caused in part by IL-10 induced reversal of DC susceptibility to NK cell activation and receptor-

mediated elimination (Alter et al., 2010). Multiple STAT transcription factors can be activated by IL-10. Concordantly with the cytotoxicity results, the IL-10 induced gene profile in NK cells showed that IL-10 increased the expression of genes involved in cytotoxicity/cell activation, although their role in NK cells has not yet been elucidated (Mocellin et al., 2004). Thus, STAT-dependent gene expression by IL-10 appears to be achieved in a combinatorial and multi-factorial manner in NK cells.

Previous experiments have demonstrated that cancer cells activate NK cells to produce IFN-γ, resulting in an increase of the anticancer activity of DC, T and NK cells (Yang and Lattime, 2003). Inappropriate up-regulation of NK cells, however, may have detrimental effects on the host. Excessive immune activation can synergize NK cell function with endogenously produced IL-10. Alternatively, IL-10 can down-modulate antigen specific T cells proliferation via MHC II down-regulation (de Waal Malefyt et al., 1991). Over activation might be controlled through a negative feedback mechanism involving IL-10 production. Although the amounts of IL-10 produced by NK cells are low compared with those produced by Th cells or monocytes, IL-10 might have a positive effect within the NK cell population via at least STAT signaling pathways.

In conclusion, IL-10 increased cytotoxicity and STAT signals. Also, we showed that IL-15 dramatically induced IL-10 secretion in human NK cells and IL-10 had an additive effect on the signaling cascade in NK cells. These results suggest that IL-10 can not only suppress immune function, but also up-regulate the initial stages of innate immune response mediated by NK cells.

## ACKNOWLEDGMENTS

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