Potent induction of IFN-γ production from cord blood NK cells by the stimulation with single-stranded RNA

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Title:

Potent induction of IFN-γ production from cord blood NK cells by the stimulation with single-stranded RNA

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Abbreviations:

TLR: Toll-like receptor, ssRNA: single-stranded RNA, APB: adult peripheral blood, CB: cord blood

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Conflict of interest:

No conflict of interest exists.

Abstract:

Natural killer (NK) cells play important roles in the innate immunity against viral infections. Although newborn infants are more susceptible to severe and recurrent viral infections than adults, the precise role of NK cells in the innate immunity against viral infections during neonatal period is not known. To clarify the functional characteristics of cord blood (CB) NK cells, we examined the capacity of CB NK cells to produce IFN-γ in response to the Toll-like receptor (TLR) ligands. We found that NK cells produced a large amount of IFN-γ by the stimulation with ssRNA, a TLR8 ligand, in the presence of IL-2, INF-α and monocytes. Surprisingly, CB NK cells produced higher amount of IFN-γ than adult peripheral blood (APB) NK cells in this condition. IL-12 produced from monocytes by the stimulation with ssRNA was indispensable for the production of IFN-γ by NK cells. NK cells in cooperation with other innate immune cells may play more important role during the neonatal period than in adults in the host defense against viral infections by high capacity of IFN-γ production to compensate immature acquired immunity.

Key words:

Innate immunity

Cord blood

Natural killer cells

IFN-γ

Introduction

The innate immune system is the first line of host defense mechanism against microbes, and the quality and magnitude of its reaction affect the subsequent adaptive immune responses. One of the reasons of increased susceptibility to the severe and recurrent viral infections in neonates than adults is more naïve nature of T cells with reduced responsiveness to the stimulation of viral antigens. On the other hand, the extent of functional difference of innate immune response between neonates and adults is not known [1, 2]. Natural killer (NK) cells are innate immune cells that play important role against viral infections and in preventing the development of tumors [3]. Actually, patients with NK cell deficiency have increased risk of mortality following the infection with herpes viruses in early childhood [4]. NK cells in cooperation with accessory cells produce a number of cytokines and chemokines in the early phase of viral infections, which play an important role in the host defense against them and lead to shape the adaptive immune responses [5, 6]. Toll-like receptors (TLRs) are pathogen-associated molecular pattern recognition receptors that trigger the signals of pathogenic intrusions for the induction of various innate immune reactions. It has been reported that NK cells also express various kinds of TLRs, and respond to their ligands [7-9]. Until now, at least 4 TLRs have been implicated to contribute in the innate

immune response to viral infections. TLR3 and TLR9 recognize short stretches of dsDNA/RNA [10] and CpG motifs of ssDNA [11], respectively. TLR7 and TLR8 have been implicated in the recognition of uridine-rich ssRNA [12]. Interaction between NK cells and accessory cells which also represent the component of the innate immune system recognizing pathogens and bridging then to adaptive immune system has been an enigma [13]. Recently, it was reported that human NK cells interact with monocytes during the innate immune response [14, 15] in which their direct contact was necessary. However, it has not been clarified if such interaction is also occurring in vivo.

In this study, we investigated IFN-γ production from cord blood (CB) NK cells in response to ssRNA stimulation, and analyzed the role of monocytes in this process to clarify the functional characteristics of CB NK cells in innate immunity against viral infections. We report here that CB NK cells can produce higher amount of IFN-γ than adult peripheral blood (APB) NK cells by TLR8 activation, and that IL-12 produced by monocytes plays an indispensible role for this reaction.

Materials and Methods

Cell preparation and culture condition:

Heparinized human 23 full-term (38-41 weeks) CB samples were collected at the time of elective Caesarean sections and normal spontaneous deliveries of healthy mothers after informed consent was obtained. All mothers had no complications including infectious diseases during perinatal period.

APB was also obtained from healthy adult volunteers aged 25 to 35. Mononuclear cells (MNC) were isolated from CB and APB by density-gradient centrifugation using LSM (Cappel-ICN Immunobiologicals, Costa Mesa, CA, USA). After the depletion of CD3+, CD19+ and CD14+ cells from MNC by using anti-CD3 monoclonal antibody (mAb)-, anti-CD19 mAb- and anti-CD14 mAb-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, NK cells were positively selected with microbeads conjugated to anti-CD56 (Miltenyi Biotec). Monocytes were positively selected from the CD3- CD19- fraction by using anti-CD14 mAb-conjugated microbeads (Miltenyi Biotec). The purity of each fraction was more than 97%. There was no significant difference in the ratio of NK cell subpopulations which were determined by expression levels of CD16+ and CD56+ in purified NK cells between CB and APB (data not shown). Also, there were no significant differences in the ratios of CD56 bright cells (17.3%±3.2% in CB and 14.7%±3.2% in APB) and of

CD56 dim cells (81.9±4.7 in CB and 84.9±3.2 in APB) between CB and APB after purification. Cells were suspended in RPMI-1640 culture medium (Gibco, Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (Gibco) and 1% gentamicin. Purified NK cells and autologous monocytes were cultured at the concentration of 1 $x10^5$ cells/ml and 1 $x10^4$ cells/ml, respectively, with or without rhIL-2 (1,000 unit/ml) (R&D Systems, Minneapolis, MN, USA), rhIFN-α (1,000 unit/ml) (PBL InterferonSource, Piscataway,NJ, USA), ssRNA40/lyovec (Invivogen, Carlsbad, CA, USA) (2 μg/ml), polyinosinic-polycytidylic acid (poly (I:C)) (25 μg/ml) (Invivogen), anti-human IL-12 antibody (10 μg /ml) (R&D Systems) and anti-human IL-18 antibody (1 μg /ml) (MBL, Nagoya, Japan) in a flat-bottom 96-well culture plate at 37 °C in a 5% CO_2 incubator. After 48 hours, the culture supernatants were harvested to measure of IFN-γ, IL-12, and IL-18 concentrations. Also, the cells were collected for flow cytometric analysis and gene expression analysis by real-time polymerase chain reaction (RT-PCR).

Flow cytometric analysis:

Fluorescein isothiocyanate (FITC)-conjugated anti-CD56 mAb, phycoerythrin (PE)-conjugated anti-CD69 mAb and PE-conjugated anti-mouse immunoglobulin (Ig) antibody were purchased from BD Pharmingen (San Jose, CA), Immunotech (Marseille,

France) and eBioscience (San Diego, CA), respectively. Anti-CD3-FITC, anti-CD19-PE, anti-CD14-PC5, anti-CD16-PE, anti-CD16-FITC, and anti-CD56-PC5 were purchased from Beckman Coulter (Miami, FL). Flowcytometric analysis was performed by using EPICS XL (Beckman Coulter).

Measurement of cytokine concentrations of culture supernatants:

Interferon (IFN)-γ concentration in culture supernatants was measured by using the Cytometric Bead Array (CBA) human Th1/Th2 kit II (BD Pharmingen, CA) according to the manufacturer's instructions. Data were analyzed using CBA software (BD Pharmingen). Sandwich enzyme-linked immunosorbent assay (ELISA) was used for the measurement of IL-12p40 (R&D Systems) and IL-18 (MBL) concentration. The optical density was measured at 450 nm with an Immunoreader NJ-2000 (Nunc, Roskilde, Denmark). All samples were run in duplicate. The sensitivity of the assay was 15 pg/ml for IL-12 and 12.5 pg /ml for IL-18, respectively.

Real-time quantitative reverse transcriptase-PCR (qRT-PCR):

Total RNA was extracted by RNAeasy micro kit (Qiagen, Hilden, Germany). Double-stranded complementary DNA (cDNA) was synthesized from total RNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA,USA). Assay-on-Demand Gene Expression Products which include PCR primers and TaqMan

probes for the measurement of IL-18 (Hs.00155517_m1), IL-12 (Hs.00233688_m1), IFN-γ (Hs.99999041_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression levels were purchased from ABI (Applied Biosystems). TaqMan assay was performed according to the manufacturer's instructions. The qRT-PCR was performed by using ABI PRISM 7700 Sequence Detector (Applied Biosystems). The gene expression levels were described by the relative ratios of the levels of target genes to those of the internal control (GAPDH gene).

Statistical analysis:

One-way ANOVA with post test (Tukey-Kramer or Dunnett Multiple Comparisons Test) were used to test for statistical significance of differences between experimental groups; *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$.

Results

CB NK cells produce higher amount of IFN-γ than adult NK cells by the stimulation with TLR-8 ligand.

NK cells produce IFN-γ, which plays an essential role in the early innate immune response against infections [16]. To examine the response of CB NK cell to viral stimulations, purified NK cells were stimulated with poly (I:C) and ssRNA, the agonists for TLR-3 and TLR-8, respectively, in vitro for 48 hours, and IFN-γ production from NK cells was investigated. Poly (I:C) and ssRNA failed to induce IFN-γ production from purified NK cells from CB and APB (Fig.1A) in agreement with a previous report [17]. It was reported that R848, an agonist of TLR7/8 induced NF-κB activation of NK cells more strongly when IL-2 or IFN-α was added [7]. Therefore, we investigated the effect of adding IL-2 and IFN-α. As shown in figure 1A, the amount of IFN-γ production of purified NK cells from both CB and APB was negligible even in the presence of IL-2 or IFN-α. It has been reported that accessory cells which include dendritic cells and monocytes/macrophages play an important role in innate immune system by providing cytokines for NK cell activation [7, 17]. As shown in figure 1B, IFN-γ production was observed both in NK cells from both CB and APB in response to TLR 3 and 8 in the presence of autologous monocytes, when IL-2 and IFN-α were added. Interestingly, CB NK cells produced significantly higher amount of IFN-γ in

response to TLR 8 than APB (Fig. 1A). IFN-γ mRNA expression levels of CB NK cells were significantly higher than those of APB NK cells in this condition (Fig. 1B). IFN-γ was preferentially detected in NK cells but almost not in monocytes by flow cytometric analysis after intracellular IFN-γ staining of these cells after culture both in CB and APB, suggesting NK cells were the main producer of IFN-γ in this culture condition (data not shown).

Effect of ssRNA on CD69 Expression of NK cells

We investigated the CD69 expression levels as an activation marker on NK cells 48 hours after the stimulation with ssRNA. The expression levels of CD69 on NK cells increased in the presence of IL-2 and IFN-α (Fig 2). On the other hand, CD69 expression levels did not show significant changes by the stimulation with ssRNA in both APB and CB even in the presence of IL-2 and IFN-α (Fig. 2). Addition of monocytes had no significant effect on CD69 expression levels in this culture condition (Fig. 2), suggesting that these cytokines act directly on NK cells and that CD69 expression levels do not correlate with the capacity of IFN-γ production.

TLR8 agonists effectively induce IL-12 production by CB and APB monocytes

IL-12 and IL-18 have been reported to play pivotal role in the induction of IFN-γ from NK cells [18, 19]. To investigate the role of IL-12 and IL-18 in CB IFN-γ production triggered by TLR8, we compared the ability of ssRNA to induce IL-12 and IL-18 production from purified monocytes. As shown in figure 3A, ssRNA induced potent IL-12 p40 production from monocytes. IL-12 production was significantly higher in CB than APB. Consistently, IL-12 mRNA expression of monocytes was enhanced by the stimulation with ssRNA, and it was induced more potently in CB monocytes than APB monocytes (Fig. 3B). Addition of IFN-α or IL-2 did not increase the production of IL-12 from monocytes (Fig. 3A), which suggests that these cytokines exert their effect on NK cells but not on monocytes for the potent IFN-γ production from NK cells. On the other hand, poly (I:C) produced very small amount of IL-12 both in CB and APB (Fig. 3A), which is compatible with the less IFN-γ production by the stimulation with poly (I:C) than with ssRNA (Fig. 1A). On the other hand, IL-18 production was not enhanced by the stimulation with ssRNA or with poly (I:C) (data not shown).

IL-12 play pivotal role in the induction of IFN-γ from NK cells

To clarify the role of IL-12 in the induction of IFN-γ from NK cells with the stimulation of ssRNA in the presence of IFN-α, IL-2 and monocytes, we investigated

the effect of adding neutralizing anti-IL-12 antibody in this culture. Profound reduction of IFN-γ production was observed in the presence of anti-IL-12 mAb (Fig. 4). This result suggests a pivotal role of IL-12 in induction of IFN-γ from both in CB and APB NK cells. Addition of anti-IL-18 mAb had no significant reduction of IFN-γ production from NK cells (Fig. 4). These results indicate that induction of IFN-γ production by NK cells is dependent on IL-12 produced by monocytes by the stimulation of TLR8 agonist, and the TLR8 agonist had direct effect primarily on monocytes but not on NK cells because NK cells could not produce IFN-γ in the absence of monocytes (Fig. 1A)

Discussion

Neonates are unduly susceptible to a wide variety of infections [20]. Viral and bacterial infections of respiratory or intestinal tract in neonates are still an important problem in developing countries [2, 21]. The major causative viruses for high morbidity and mortality during infancy include herpes simplex virus (HSV), cytomegalovirus, varicella-zoster virus and respiratory syncytial virus [22-24]. Lower ability of cellular immune response against viral antigens and the defect of antigen-inexperienced immune repertoire against them may be one of the reasons why virus infections occur more frequently and more severely in neonates. On the other hand, the potency of innate immune response against viruses in neonates has not been clarified, yet.

In the innate immune system, NK cells play important roles against viral infections by producing various cytokines including IFN-γ and preferentially killing infected cells after the recognition of infected cells by various NK receptors [25]. In lymphocytic choriomenigitis virus (LCMV) infection in mice, NK cells are key effector cells to eradicate the virus by the production of IFN-γ in the early phase of infection [26], and IFN-γ production in this model is dependent on IL-12 which is produced by accessory cells [27]. In murine bacterial infection models, IL-12 produced by accessory cells stimulates NK cells to produce IFN-γ which in turn helps control of the infection until an adaptive immune response finally eliminates the pathogens [28, 29]. In humans,

the patients with NK cell deficiency suffer from recurrent and severe viral infections, especially herpes viruses [30, 31]. Therefore, it is important to know the role of NK cells in host defense mechanisms against viral infections during the neonatal period.

Recently, it has been reported that NK cells also respond to conserved microbial products through TLRs [7, 9, 17]. In this study, we focused on the NK cell response against TLRs which reacts with virus associated molecules. Hart et al. [7] reported that poly (I:C), the TLR3 ligand, directly activated resting NK cells and induced their cytotoxicity. On the other hand, IFN-γ production was not detected from NK cells after poly (I:C) stimulation [7]. In our study, although IFN-γ production could not be detected by poly (I:C) stimulation, small amount of IFN-γ production was observed by the stimulation of poly (I:C) in the presence of IL-2 (Fig. 1A). This indicates a contribution of IL-2 for the induction of IFN-γ production by TLR3 agonists. Usually, there are various immune cells and various kinds of cytokines at the site of viral infections in vivo. It was reported that IFN-α production occurs within a few hours after the viral infections from fibroblasts [32]. There has been accumulating evidence that IFN-α contributes to induce Th1 cells, in addition to the direct anti-viral effect [33]. IFN-α is reported to activate CD4 and CD8 T cells which play an important role against viral infections through the induction of IFN-γ, chemokines and other cytokines [34, 35].

On the other hand, it was reported that TLR ligand stimulation cause robust IL-2 production from CD8 T cells, which suggests the presence of IL-2 at the site where innate immune response is activated [36]. In our study, NK cells produced a high amount of IFN-γ by the stimulation with TLR8 ligand in the presence of IL-2, IFN-α and monocytes, although the augmented effect of IFN-α was remarkable in CB NK cells but not in APB NK cells. This is the first report on functional characteristics of CB NK cells activated by TLRs. In addition, we demonstrated that CB NK cells produced higher amount of IFN-γ, which may play important role in host defense against viral infections in neonatal period.

It was reported that CB monocytes of full-term human newborns expressed normal amounts of TLRs [37, 38], and the R-848 and 3M-003 (TLR7/8 agonist), and 3M-002 (TLR8 agonist) were able to stimulate IL-12 production by accessory cells including monocytes [39]. In this report, we showed that IL-12 production by monocytes with the stimulation of ssRNA was indispensable for IFN-γ production from NK cells, because IFN-γ production from NK cells was abrogated by the addition of anti-IL-12 mAb (Fig. 4). Higher production of IL-12 from monocytes in CB monocytes than APB monocytes may be one of the mechanisms of increased IFN-γ production from CB NK cells in this condition (Fig. 1, 3). IFN-γ production from NK cells may

help to initiate a T helper (Th)1-type adaptive immune response which is required for successful elimination of most viral pathogens [40, 41].

It has been reported that IFN-γ production is strongly induced by the stimulation with IL-12 and IL-18 [41]. Previously, we [42] and Satwani et al. [43] demonstrated that CB NK cells produced higher amount of IFN-γ with the stimulation of IL-12 and IL-18 than APB NK cells [42, 43]. CB NK cells showed more CD69 expression following IL-12 andIL-18 stimulation [42]. IL-12 alone was inefficient for the induction of IFN-γ production and optimally synergizes with cytokines such as IL-2 and IL-18 [44]. On the other hand, we could not find any role of IL-18, in which TLR8 agonist was used for IFN-γ induction. Purified monocytes from both CB and APB failed to induce IL-18 production after the stimulation with ssRNA (data not shown). And we could not detect IL-18 mRNA in monocyte after the stimulation (data not shown). Furthermore, anti-IL-18 and anti-IL-12 had no further blocking effect on IFN-γ production from NK cells than anti-IL-12 alone (Fig. 4). Therefore, the NK cell response in this condition seems to be IL-18-independent.

The expression levels of CD69 on CB NK cells were augmented after stimulation with IL-2 and IFN-α (Fig. 2). On the other hand, IL-12 produced by monocytes after stimulation with ssRNA has no effect on CD69 expression in both adult

and CB NK cells (Fig. 2). Although CD69 has been known to be one of the activation markers of NK cells as well as T cells, increase of CD69 expression levels on NK cells do not seem to have clear correlation with the capacity of IFN-γ production as previously reported [45, 46].

In contrast to the immature acquired immune system in human neonates and infants, which is the cause of increased susceptibility to infections by a wide range of pathogens, innate immune system to produce IFN-γ by NK cells may play more important role in them than adults in preventing viral pathogens in concert with accessory cells.

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Figure legends:

Figure 1. Preferential induction of IFN-γ production from CB NK cells by ssRNA.

IFN-γ productions from CB NK cells and APB NK cells were investigated after incubation for 48 hours with or without IL-2, IFN-α, poly (I:C) and ssRNA. Purified NK cells were incubated at the concentration of $1x10^5$ cells/ml without and with purified monocytes ($1x10^4$ cells/ml) (A). Cell free culture supernatants were collected and IFN-γ concentrations were measured by using CBA cytokine kit II. Data represent means and standard deviation (SD). (n=5). The IFN-γ mRNA expression levels of CB NK cells and APB NK cells were determined by qPCR after incubation for 48 hours with or without rhIL-2, IFN-α and ssRNA in the presence of monocytes (B). The gene expression levels were described by the relative ratios of the levels of IFN-γ genes to those of the internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. A: Closed bars and open bars represent CB and APB, respectively. B: Closed squares and open squares represent CB and APB, respectively. Data represent means and SD (n=4).

Figure 2. CD69 expression on NK cells.

Freshly isolated NK cells from CB and PAB were cultured with or without monocytes for 48 hours in the presence or absence of IL-2, IFN-α and ssRNA. Expression levels of CD69 on $CD56^+$ cells were analyzed by using flow cytometer. Closed bars and open bars represent CB and APB, respectively. Data represent means and SD of 5 independent experiments.

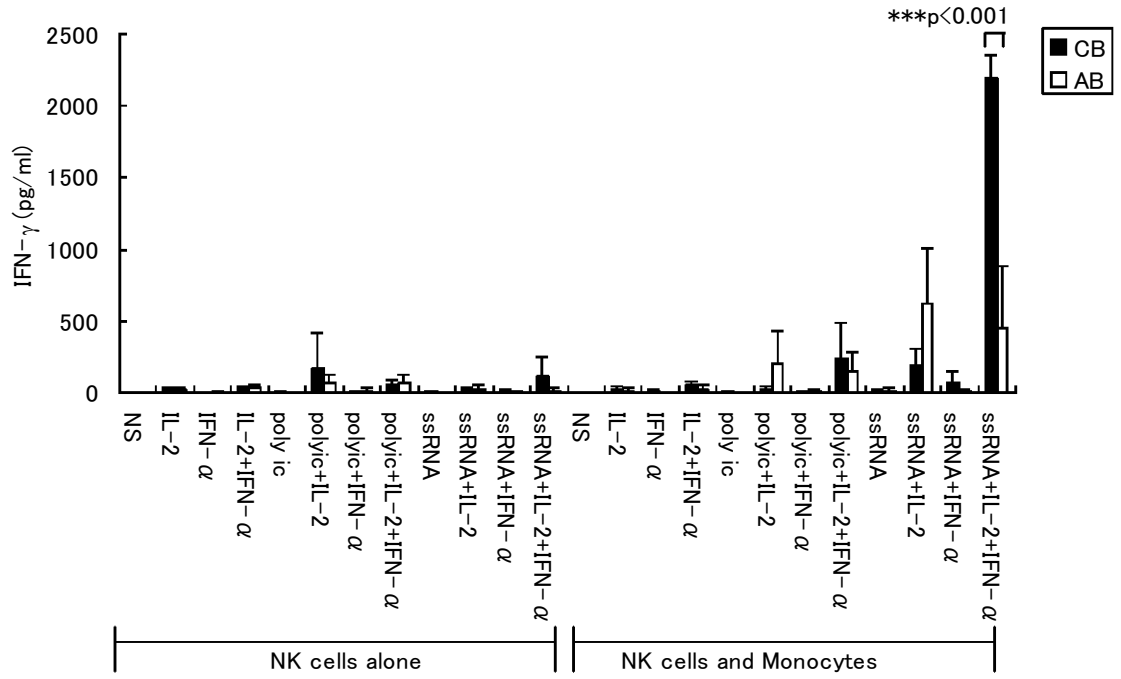
Figure 3. IL-12 production from monocytes by the stimulation with ssRNA.

Purified Monocytes from CB and APB were cultured in a 96-well plate (1×10^5 cell/ml) with or without ssRNA40, poly (I:C), IL-2 and IFN-α at 37°C in a 5% CO_2 incubator for 48 hours. IL-12 p40 production was measured in culture supernatants by ELISA (A). Closed bars and open bars represent CB and APB, respectively. Data represent means and SD (n=5). IL-12 p40 mRNA expression levels of cultured monocytes stimulated with ssRNA was measured by q-PCR (B). Closed squares and open squares represent CB and APB, respectively. The gene expression levels were described by the relative ratios of the levels of target genes to those of the internal control (GAPDH) gene. Data represent means and SD (n=4).

Figure 4. Defect of IFN-γ production from NK cells by the stimulation with ssRNA in the presence of anti-IL-12 antibody. IFN-γ concentration was measured for cell free culture supernatants of CB and APB NK cells after the incubation for 48 hours in the presence of IL-2, IFN-α, ssRNA, and monocytes, with or without anti- IL-12 and anti-IL-18 antibodies. Closed bars and open bars represent CB and APB, respectively. Data represent means and SD (n=5).

Figure 1

A


***p<0.001
CB
AB
IFN-γ (pg/ml)
2500
2000
1500
1000
500
0
NS
IL-2
IFN-α
IL-2+IFN-α
poly ic
polyic+IL-2
polyic+IFN-α
polyic+IL-2+IFN-α
ssRNA
ssRNA+IL-2
ssRNA+IFN-α
ssRNA+IL-2+IFN-α
NS
IL-2
IFN-α
IL-2+IFN-α
poly ic
polyic+IL-2
polyic+IFN-α
polyic+IL-2+IFN-α
ssRNA
ssRNA+IL-2
ssRNA+IFN-α
ssRNA+IL-2+IFN-α
NK cells alone
NK cells and Monocytes

B

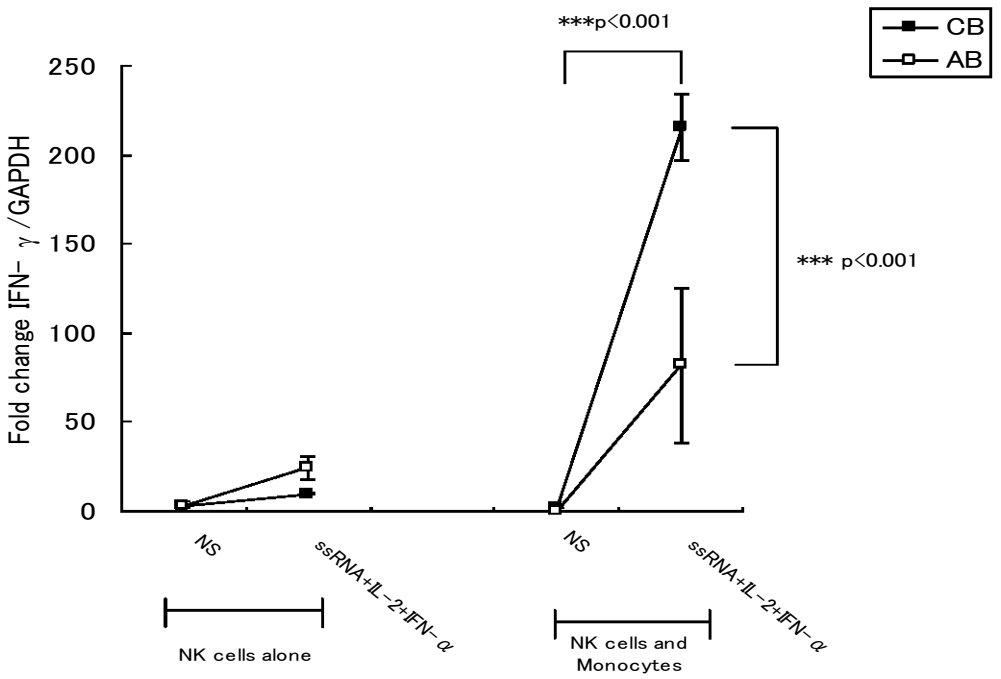

***p<0.001
CB
AB
Fold change IFN-γ/GAPDH
250
200
150
100
50
0
*** p<0.001
NS
ssRNA+IL-2+IFN-α
NS
ssRNA+IL-2+IFN-α
NK cells alone
NK cells and Monocytes

Figure 2

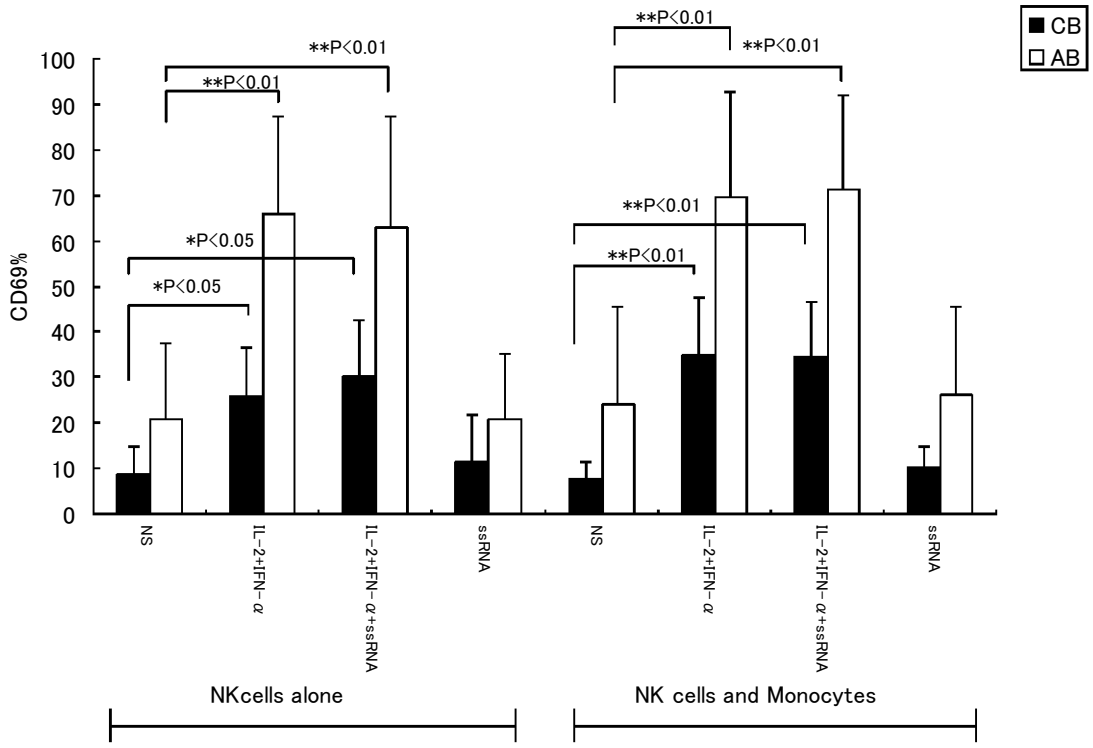
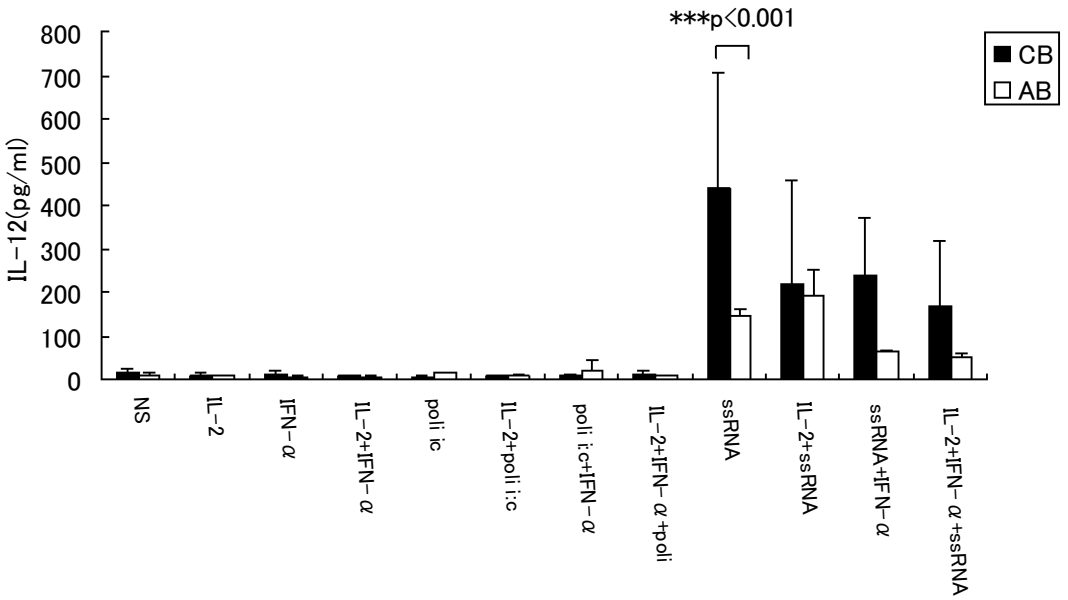

CB
AB
CD69%
100
90
80
70
60
50
40
30
20
10
0
**P<0.01
**P<0.01
**P<0.01
**P<0.01
*P<0.05
**P<0.01
*P<0.05
**P<0.01
NS
IL-2+IFN-α
IL-2+IFN-α+ssRNA
ssRNA
NS
IL-2+IFN-α
IL-2+IFN-α+ssRNA
ssRNA
NKcells alone
NK cells and Monocytes

Figure 3

A


***p<0.001
CB
AB
IL-12(pg/ml)
800
700
600
500
400
300
200
100
0
NS
IL-2
IFN-α
IL-2+IFN-α
poli ic
IL-2+poli i:c
poli i:c+IFN-α
IL-2+IFN-α+poli
ssRNA
IL-2+ssRNA
ssRNA+IFN-α
IL-2+IFN-α+ssRNA

B

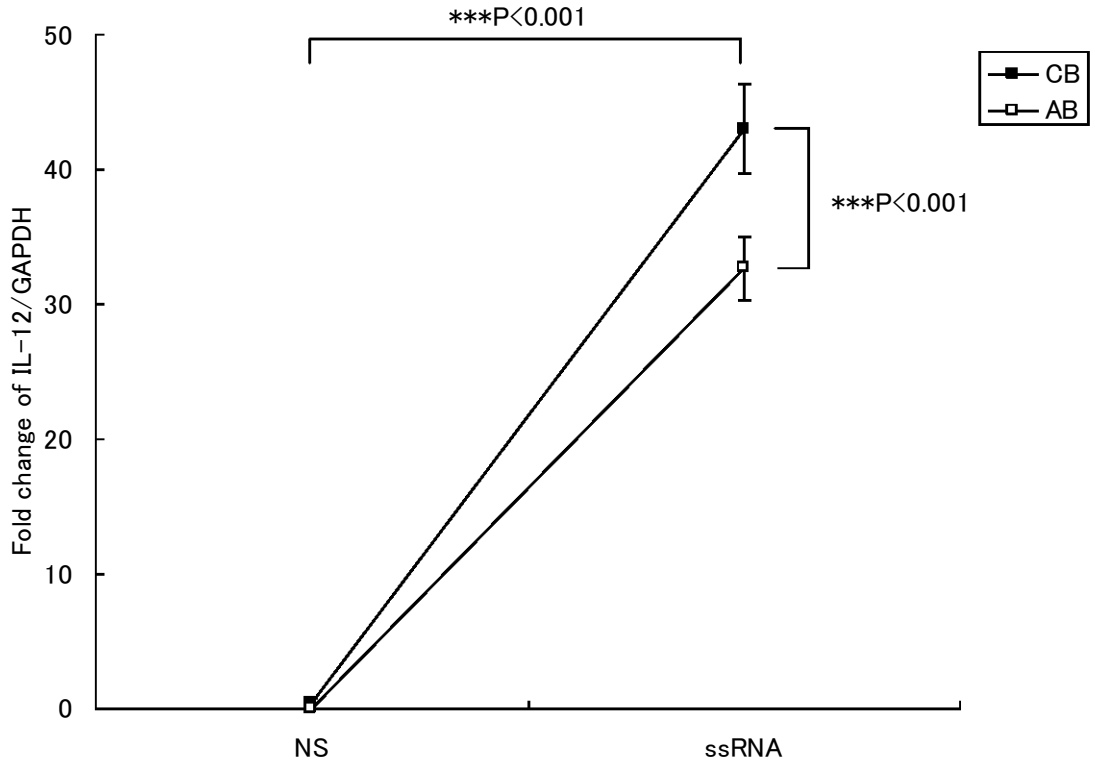
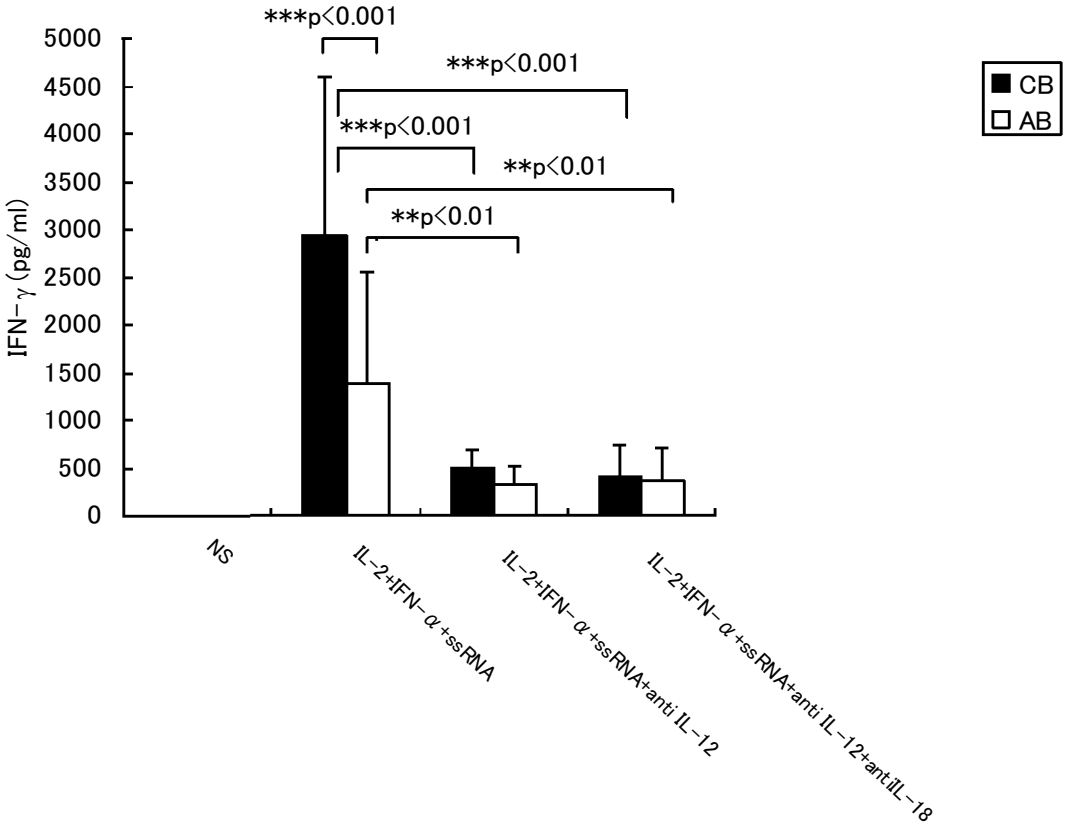

***P<0.001
***P<0.001
CB
AB
Fold change of IL-12/GAPDH
50
40
30
20
10
0
NS
ssRNA

Figure 4


***p<0.001
***p<0.001
***p<0.001
**p<0.01
**p<0.01
IFN-γ (pg/ml)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
CB
AB
NS
IL-2+IFN-α+ssRNA
IL-2+IFN-α+ssRNA+anti IL-12
IL-2+IFN-α+ssRNA+anti IL-12+anti IL-18