

## Article

# 17 $\beta$ -Estradiol Suppresses Cytotoxicity and Proliferative Capacity of Murine Splenic NK1.1<sup>+</sup> Cells

Sha Hao<sup>1</sup>, Pengfei Li<sup>1</sup>, Junli Zhao<sup>1</sup>, Yali Hu<sup>2,3</sup> and Yayi Hou<sup>1,3,4</sup>

In order to clarify the effects of 17 $\beta$ -estradiol (E2) on natural killer (NK) cells and the possibly regulatory mechanisms, we obtained highly purified and viable NK cells from C57BL/6J mouse spleen by a magnetic cell sorter (MACS). These cells were treated with E2 and then their cytotoxicity and proliferative capacity were examined. To further investigate the mechanisms on the effect of E2 on NK cells, expressions of activation-associated markers (CD69, CD122) and inhibitory receptors (CD94, Ly49), and intracellular cytokine production were analyzed. At last, we performed the cDNA microarray to explore the possible involved genes. We found that E2 could suppress NK cell cytotoxicity and proliferative capacity *in vitro*. E2 reduced NK cell cytotoxicity and proliferative capacity, which may be through influencing the phenotypes and cytokine expression of NK cells, mainly involving CD94 and IFN- $\gamma$ . Furthermore, regulation of Stat4, Fyn, Sh2d1a, Eat2, Cd244, Irf1, Runx1, Irf7, Irf5, Esrra and Nr5a1 genes may be related to the cytotoxicity, proliferation and cytokine production of E2-mediated purified NK cells. *Cellular & Molecular Immunology*. 2008;5(5):357-364.

**Key Words:** 17 $\beta$ -estradiol (E2), natural killer cell, proliferative capacity, cytotoxicity

## Introduction

The fetal tolerance is notably dependent on the maternal immunocompetence during the pregnancy (1). During this period, the changes in number, phenotype and activity of NK cells were observed. Peripheral NK cells from pregnant women exhibit decreased lytic activity compared to NK cells from controls. Moreover, the dynamic changes occurred in lymphocyte populations in early pregnancy (2). The dominant lymphocytes in human and murine implantation sites are transient, pregnancy-associated uterine natural killer (uNK) cells, which account for about 70% of resident lymphocytes in human decidual cell suspensions (3). Thus, uNK cells may play a role in implantation and pregnancy, at

least in early gestation (4). Otherwise, uNK cells may derive predominantly from a subset of peripheral NK cells, which were recruited to the decidua of the uterus of pregnant woman under hormonal influence (5). This suggests that both uNK cells and peripheral NK cells may be hormonally regulated (6) since the levels of sex hormones are markedly changed during implantation and maintenance of pregnancy.

17 $\beta$ -estradiol (E2), an active estrogen, is one of the important sex hormones during pregnancy. It exerts many genomic and nongenomic effects through binding to the intracellular or membrane receptor proteins (7). Estrogen receptor  $\alpha$  (ER $\alpha$ ) and ER $\beta$  have been recently identified in mouse splenic NK cells (8). It suggests that E2 may be involved in the regulation of NK cell functions.

Many investigations have been conducted on estrogens influencing a variety of immune functions using *in vivo* or *in vitro* models, but different results were obtained. Sorachi K, et al. (9) found that estrogen could enhance the *in vitro* cytotoxicity in YT-N17 (a human NK-like cell line). Seaman et al. (10) reported that estrogen decreased the NK activity *in vivo*. These inconsistent results may attribute to the difference in the sources and purity of NK cells. Our

<sup>1</sup>Immunology and Reproductive Biology Lab of Medical School & State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, China;

<sup>2</sup>The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing 210008, China;

<sup>3</sup>Jiangsu Key Laboratory of Molecular Medicine, Nanjing University, Nanjing 210093, China;

<sup>4</sup>Corresponding to: Dr. Yayi Hou, Immunology and Reproduction Biology Lab, Medical School, Nanjing University, Nanjing 210093, China. Tel: +86-25-8368-6441, Fax: +86-25-8368-6441, E-mail: yayihou@nju.edu.cn

Received Jul 10, 2008. Accepted Sep 18, 2008.

**Abbreviations:** NK cell, natural killer cell; uNK cell, uterine natural killer cell; E2, 17 $\beta$ -estradiol; ER, Estrogen receptor  $\alpha$ ; IFN- $\gamma$ , interferon  $\gamma$ ; TNF- $\alpha$ , tumour necrosis factor  $\alpha$ ; PI, propidium iodide; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; CFDA-SE, carboxyfluorescein diacetate, succinimidyl ester; FCS, fetal calf serum; RIA, radioimmunoassay.

previous study also found that E2 increased the number of NK cells, but reduced their cytotoxicity *in vivo* (11). However, in organism the treatment of E2 not only acts on NK cells but also exerts on other cells to bring surroundings around NK cells.

To clarify the changes in the function of NK cells related to sex hormone during pregnancy, we used highly purified murine splenic NK cells. The purified NK cells, isolated by using anti-NK cell (DX5) Micro Beads, were treated with E2 (a representative estrogen during pregnancy), and cytotoxicity of E2 on purified NK cells were examined. In addition, expressions of activation-associated markers (CD69, CD122) (12) and inhibitory receptors (CD94, Ly49) (13), and intracellular cytokine production were analyzed. The cDNA microarray was conducted to further investigate the mechanisms.

## Materials and Methods

### *Mice*

All the experiments were performed by using 4-5 week female mice of C57BL/6J (B6) purchased from Model Animal Research Center of Nanjing University. This strain expresses the NK1.1 antigen on the surface of NK cells (14). All the animals were housed five per cage at 24°C in a humidity-controlled room with 12 h light/dark photoperiod (light on at 8:00 and off at 20:00), constant access to water and chow. Mice were allowed to acclimatize for at least 1 week prior to using in experiments.

### *Cell culture*

The NK-sensitive murine lymphoma cell line YAC-1 was used as the target cells for the cytotoxicity assay. The cells were cultured in complete RPMI 1640 medium (HyClone), supplemented with 10% heat-inactivated FCS, 20 mM Hepes, 2 mM L-glutamine, 2 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/L streptomycin at 37°C in 5% CO<sub>2</sub>.

### *Reagents*

Mouse anti-NK cell (DX5) Micro Beads were purchased from Miltenyi Biotec, Germany. The following antibodies used for flow cytometry: anti-NK1.1-APC, CD3-PE-CY5.5, CD69-FITC, CD94-FITC, Ly49-FITC, IFN- $\gamma$ -FITC and isotype control antibodies were purchased from eBioscience. Anti-TNF- $\alpha$ -FITC was obtained from Becton and Dickinson Company (San Jose, CA, USA). E2, collagenase D, MTT, PI, monensin and saponin were purchased from Sigma Chemical Co. (St., Louis, Mo, USA). CFSE was obtained from Marker Gene Technologies, Inc. Anti-CD122-PE and dead cell discriminator (DCD) were purchased from Caltag Laboratories. PMA and ionomycin were obtained from Alexis Biochemicals and Annexin V/PI kit purchased from Bender.

### *Assessment of plasma sex steroids*

E2 level in fetus calf serum (FCS) used in all experiments was determined by radioimmunoassay (RIA).

### *NK cell preparation*

Mice were killed by cervical dislocation and spleens were removed aseptically. Single-cell suspensions were prepared by teasing with sterile forceps, digested with 1 mg/ml collagenase D for 30 min at 37°C, and then filtrated through the 100-mesh and 200-mesh cell strainer. The filtrate containing single cells was spun down at 1,500 rpm for 5 min. The obtained single splenic cells were treated with ACK lysing buffer (0.15 M NH<sub>4</sub>Cl, 1.0 mM KHCO<sub>3</sub>, 0.1 mM EDTA, pH 7.2) for 5 min to lyse the erythrocytes, and then washed thrice with RPMI 1640 medium containing 10% FCS. NK cells were enriched by magnetic positive selection using anti-DX5 microbeads following the manufacturer's protocol. The purity of NK cells was assessed by flow cytometry using mAb anti-NK1.1-APC and the cell viability was determined by trypan-blue exclusion and AnnexinV/PI staining.

### *Treatments of NK cells with E2*

Freshly purified DX5 positive NK cells were cultured in 96-well flat-bottom plates (1 × 10<sup>5</sup> cells/well) for 24 h with RPMI 1640 medium supplemented with 10% FCS, 20 mM Hepes, 2 mM L-glutamine, 2 mM sodium pyruvate, 50 mM  $\beta$ -mercaptoethanol, 100 U/ml penicillin G and 100 mg/L streptomycin in the absence or presence of E2. All assays were performed in triplicates.

The experiment was designed for four groups according to the concentration of E2: control group, low dose group (10 nM), middle dose group (100 nM) and high dose group (1  $\mu$ M). E2 was dissolved in 100% ethanol at 10 mM, then diluted in RPMI 1640 medium to a concentration of 10  $\mu$ M and stored at -20°C until use. The final concentration of ethanol was less than 0.1% and considered to be nontoxic to the NK cells.

### *NK cell cytotoxicity assay*

Cytotoxic activity against YAC-1 cells is a characteristic function of NK cells. The cytotoxicity of NK cells was determined as previously described (15). Briefly, the YAC-1 cell line was used as target cell and labeled with 5.0  $\mu$ M CFDA-SE for 8 min at 37°C in 5% CO<sub>2</sub>. CFSE was dissolved in DMSO at 5 mM, and then diluted in RPMI1640 medium to a final concentration of 5  $\mu$ M until use. After labeled, the cells were washed thrice and resuspended in RPMI 1640. The effect/target ratios of 100, 50, 25, 12.5 and 6.25:1 were set-up. The target cells of each sample were 10<sup>4</sup> cells. Every sample was mixed to a final volume of 200  $\mu$ l in complete medium in 96-well flat-bottom plate and incubated at 37°C in 5% CO<sub>2</sub> for 4 h. Then samples were put in an ice water bath and 25  $\mu$ l PI (100  $\mu$ g/ml) was added in and incubated for 5 min in order to label the DNA of the dead cells. The dead target cells were analyzed within 60 min.

All samples were analyzed on a FACSCalibur (Becton Dickinson, San Diego, CA) using the software Cell Quest for the acquisition and data analysis. During data acquisition, a "live cell gate" was set on the CFSE-stained target cell population using an FL1-histogram, 5,000 target events were collected. For data analysis, the CFSE-stained target cells

were further gated in an FL1/FL3 dot plot, including all living and dead target cells. CFSE<sup>+</sup>/PI<sup>+</sup> cells were considered as dead target cells. Percentage of specific target cell death (cytotoxicity) was then expressed as:

$$\frac{\text{dead targets in the sample (\%)} - \text{spontaneously dead targets (\%)}}{100 - \text{spontaneously dead targets (\%)}} \times 100$$

#### NK cell proliferative capacity assay

Cell proliferative capacity was determined by MTT assay. After purified NK cells were treated with different concentrations of E2 for 24 h, 25  $\mu$ l of 0.5% MTT dissolved in PBS was added to each well containing 100  $\mu$ l cells ( $5 \times 10^4$ ) in 96-well flat-bottom plates for 4 h. The blue crystals were dissolved by the addition of 100  $\mu$ l 20% SDS at 37°C overnight. An automated microtiter plate reader (Bio-Rad Model 550) was used to measure the light absorbance at 570 nm. Additionally, DCD, which can label dead cells by integrating with DNA, was used to distinguish non-viable cells from viable cells. After these purified NK cells were treated with different concentrations of E2 for 24 h, 1  $\mu$ l DCD was added to each sample and proportions of dead cells were detected by flow cytometry using an FL3-histogram.

#### Flow cytometric analysis of phenotypes

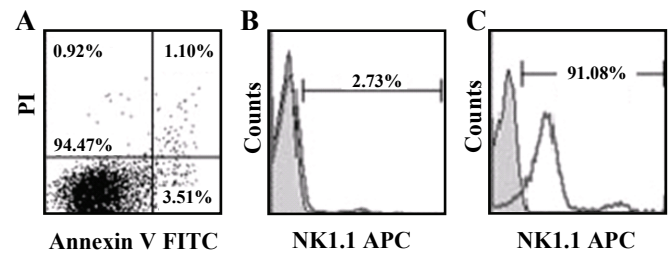
Surface phenotypes were identified by using mAbs in conjunction with two-color immunofluorescence tests. NK cells treated with E2 were incubated with FITC-, PE- and PE-CY5.5-conjugated anti-mouse antibodies for 30 min at 4°C. The cells were washed twice with FACS wash buffer (PBS + 0.1% NaN<sub>3</sub> + 1% BSA). A total of  $10^4$  cells were gated by forward scatter and side scatter. Then CD3-PE-CY5.5 negative cells were further gated, because NK cells are NK1.1<sup>+</sup>CD3<sup>-</sup> in the C57BL/6J mouse strain and generally DX5<sup>+</sup>CD3<sup>-</sup> in other mouse strains (14). So these subpopulations were analyzed for the expression of CD69, CD122, CD94 and Ly49 using an FL1 or FL2-histogram. Mean fluorescence intensity (MFI) was measured by a FACSCalibur. Data analysis was performed using the Cell Quest Software (Becton Dickinson, San Diego, CA).

#### Detection of IFN- $\gamma$ and TNF- $\alpha$ by intracellular flow cytometry

Following 12 h of treatment with E2, the purified NK cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for the next 12 h (16). Monensin (1.7  $\mu$ g/ml) was added to block cytokine secretion. The harvested cells were stained for surface expression of CD3, and then fixed for 25 minutes at room temperature in 4% paraformaldehyde and permeabilized in 0.1% saponin. After two washes with PBS buffer containing 0.1% saponin, the cells were stained with FITC-conjugated anti-mouse IFN- $\gamma$  and TNF- $\alpha$  for 30 min at 4°C. Intracellular cytokine levels in an FL1/FL3 dot plot were analyzed using CellQuest Software. Assay was performed in triplicates.

#### Purification of NK cells and microarray analysis

In order to detect the possible genes involved in the



**Figure 1. Characterization of cell viability and composition of purified NK cells.** (A) Purified DX5<sup>+</sup> NK cells were stained by FITC-conjugated Annexin V and PI to distinguish early apoptotic cells and dead cells after enriched by magnetic positive selection. Percentage of early apoptotic cells were shown in the low right region, dead cells in the up right region, and live cells in the low left region (94.47% live cells). (B) and (C) The purities of NK cells before and after isolated. The cells were stained by NK1.1 and analyzed by flow cytometry. The cells were first gated by forward scatter and side scatter. The percentages of NK1.1<sup>+</sup> cells in splenic cells and purified NK cells were shown in the histogram.

E2-mediated purified NK cell cytotoxicity, the cDNA microarray analysis was performed for the chosen two groups: control and high concentration E2 group (1  $\mu$ M). The purified NK cells were obtained according to the methods described above. Then control and E2 (1  $\mu$ M) treated NK cells were treated with 1 ml BIOzol reagent (BioFlux). RNA isolation and the quality were checked by spectrophotometry and oligonucleotide microarray analysis (36k Mouse Genome Array) was performed by CapitalBio Corp., Beijing, China.

#### Statistical analysis

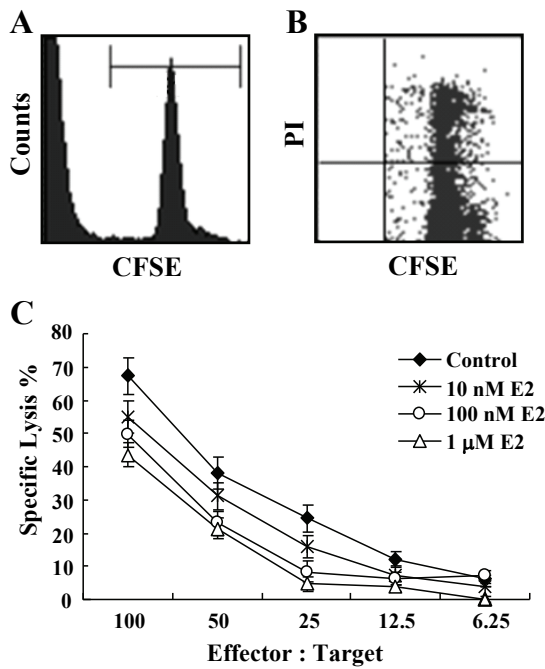
Results were shown as mean  $\pm$  standard deviation (SD). Statistical significance was assessed by one way analysis of variance (ANOVA) and the ANOVA post-hoc Bonferroni test by the software of SPSS 12.0. A  $p < 0.05$  was considered to be statistically significant. All experiments were performed more than three times and similar results were observed every time.

## Results

#### Concentration of E2 in FCS and cell purity of isolated NK cells

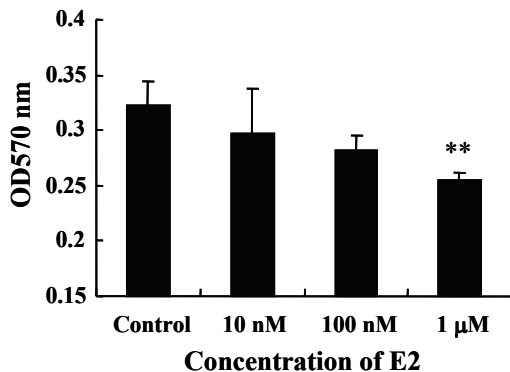
To eliminate the interference of FCS-sourced E2 in the experiments, concentration of E2 in FCS was detected by RIA prior to application. The result showed that E2 content in our used FCS was less than 22.7 nM. Ten percent FCS were used in our experiments, so the final concentration of E2 was only 2.27 nM. It was extremely low so as to ignore the effect of E2 to the FCS in our experiments.

In our study, NK cells from B6 mouse spleen were performed using a magnetic cell sorter (MACS). The cell viability of the obtained NK cells was consistently 90~95% with determination by trypan-blue exclusion or Annexin V/PI staining (Figure 1A). The splenic cells and freshly purified

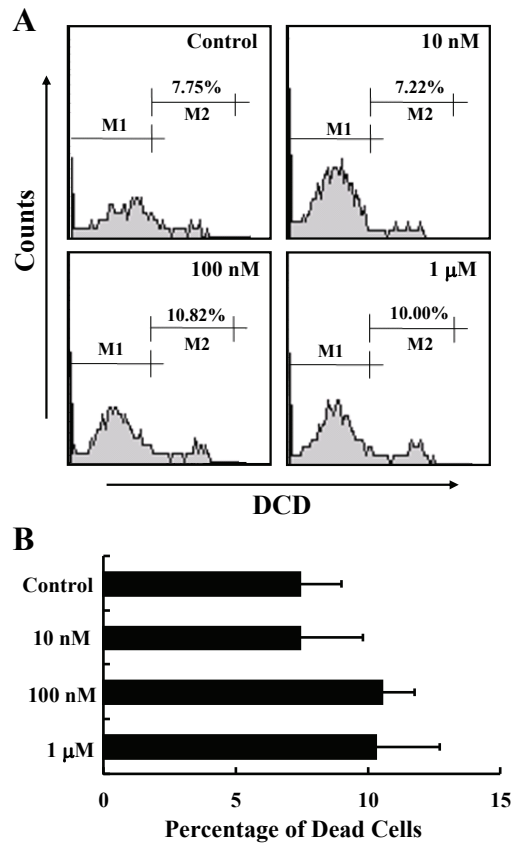


**Figure 2. E2 treatment suppresses NK cell cytotoxicity.** (A) CFSE positive target cells were gated in an FL1 histogram. (B) The target cells were further gated in an FL1/FL3 dot plot showing both live and dead target cells. (C) Specific lysis of NK cells treated with various concentrations of E2 at different E/T ratios. Data represent the mean percentage specific cytotoxicity from three separate experiments with similar findings.

NK cells were stained with APC-conjugated anti-NK1.1 antibody. NK1.1<sup>+</sup> cells of splenic cells and purified NK cells were 2.54 ± 1.14% and 89.85 ± 2.67%, respectively. We used NK1.1<sup>+</sup> cells of over 87% purity for all *in vitro* experiments (Figures 1B and 1C).



**Figure 3. E2 suppresses proliferative capacity of purified NK cells.** NK cells were cultured with various concentrations of E2 (0 nM, 10 nM, 100 nM and 1 μM) for 24 h. Then MTT assay was used to detect the proliferative capacity of each group. Data represent the mean ± SD of four parallel wells from three independent experiments. \*\**p* < 0.01 vs the control group.



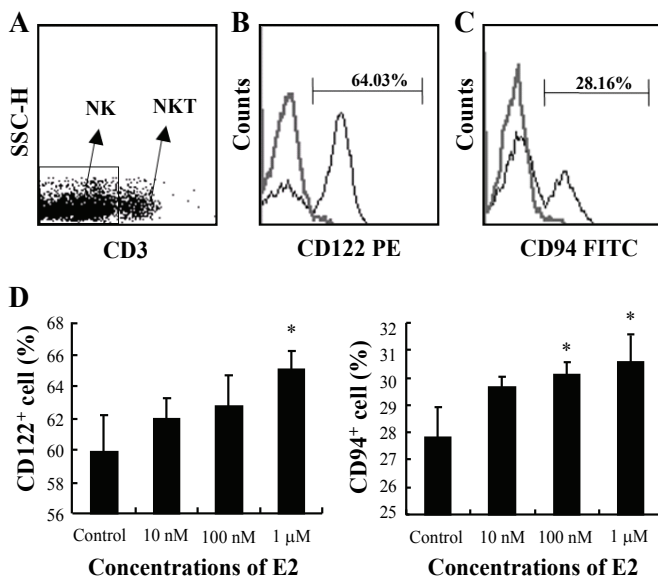
**Figure 4. E2 does not affect NK cell viability.** After various concentrations of E2 (0 nM, 10 nM, 100 nM and 1 μM) treated the purified NK cells for 24 h, DCD was used to distinguish non-viable cells from viable cells by using flow cytometry. (A) Live cells (M1) and dead cells (M2) were shown in the histograms. (B) The results were analyzed by ANOVA test. Data represent the mean ± SD of triplicate samples.

*E2 inhibites NK cells cytotoxicity*

The effect of E2 on purified NK cell cytotoxicity was examined using a novel flow-cytometric assay as previously described. Our results indicated that E2 inhibited the NK cell cytotoxicity from low dose group (10 nM) to high dose group (1 μM) compared to the control group in a dose-dependent manner. The cytotoxicity at 100 : 1 effector/target ratios of control group and each E2 treated group were 67.29 ± 5.42%, 54.91 ± 4.98%, 49.76 ± 4.03% and 43.53 ± 3.75%, respectively (Figure 2).

*E2 suppresses NK cell proliferation but has no effect on cell viability*

The effect of E2 on the proliferative capacity of NK cells was first detected by MTT assay. As shown in Figure 3, E2 can reduce the proliferative capacity in a dose-dependent manner. The viabilities of NK cells were slightly increased after incubation with 10 nM E2 (0.32 ± 0.04), but not statistically significant. The proliferative capacity of NK cells was significantly reduced after exposure to 1 μM E2 (0.25 ± 0.01) compared with the control group (0.29 ± 0.02) (*p* < 0.05)

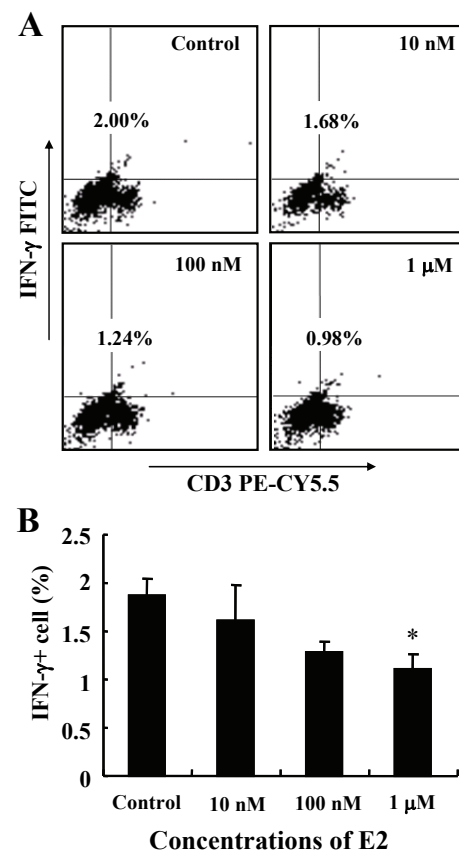


**Figure 5. E2 affected the expressions of NK cell surface molecules.** (A) CD3<sup>+</sup> NK cells were gated. (B) and (C) The percentages of CD3<sup>+</sup>CD122<sup>+</sup> and CD3<sup>+</sup>CD94<sup>+</sup> NK cells in control group were shown. (D) The results were analyzed by ANOVA test. Data represents the mean  $\pm$  SD of triplicate samples. \* $p < 0.05$ , vs the control group.

(Figure 3). Subsequently, to exclude the decreased proliferative capacity of NK cells ascribing to the increased dead cells, we further examined proportions of dead cells by DCD assay after NK cells were treated with E2 for 24 h. The percentage of dead cells in the control group and three treatment groups were  $7.44 \pm 1.56\%$ ,  $7.45 \pm 2.34\%$ ,  $10.53 \pm 1.25\%$ , and  $10.28 \pm 2.42\%$ , respectively. Significant differences were not observed between control and experiment groups (Figure 4).

#### E2 changes the expressions of NK cell surface molecules

From the above results, we found that E2 can suppress the cytotoxicity and proliferative capacity of NK cells. Furthermore, we want to know how E2 influenced the cytotoxicity of NK cells. Then we investigated two activation-associated cell surface molecules, CD69 (very early activation antigen) and CD122 (IL-2/IL-15R $\beta$ ), and two inhibitory receptors, CD94 and Ly49 on NK cells. Our results showed that E2 had no effect on expression of activation molecule CD69 (data not shown). The proportion of another NK-cell activation molecule (CD122) slightly increased with treatment of E2, and only the high concentration group (1  $\mu$ M) increased significantly ( $p < 0.05$ ),  $65.05 \pm 1.06\%$  (E2) versus  $59.85 \pm 2.34\%$ . The inhibitory receptor CD94 was activated and higher expressed after NK cell exposure to E2 compared to the control group,  $29.67 \pm 0.45\%$  (10 nM,  $p > 0.05$ ),  $30.09 \pm 0.36\%$  (100 nM,  $p < 0.05$ ),  $30.58 \pm 0.95\%$  (1  $\mu$ M,  $p < 0.05$ ) versus  $27.83 \pm 1.1$  (Figure 5). Whereas, Ly49, murine NK cells inhibitory receptors for class I MHC had no statistical significance (data not shown).



**Figure 6. Flow cytometric analysis of intracellular cytokines IFN- $\gamma$ .** (A) Cells were stained with fluorescent antibodies as described. The percentages of CD3<sup>+</sup>IFN- $\gamma$ <sup>+</sup> were analyzed by flow cytometry. (B) The results were analyzed by ANOVA test. Data represents the mean  $\pm$  SD of triplicate samples. \* $p < 0.05$ , vs the control group.

#### E2 inhibits cytokine secretion

It had been reported that cytokines, IFN- $\gamma$  and TNF- $\alpha$ , secreted by NK cells play a pivotal roles in the activation and maturation of NK cells (44). Thus, we examined the effect of E2 on intracellular cytokines. Result showed that high dose (1  $\mu$ M) of E2 inhibited IFN- $\gamma$  expression ( $1.1 \pm 0.165\%$  vs  $1.875 \pm 0.177\%$  in control group,  $p < 0.05$ ) (Figure 6). No statistical significance was observed in TNF- $\alpha$  expression (data not shown).

#### Microarray analysis

To determine which gene might be involved in the cytotoxicity and proliferation of NK cells treated with E2, we used purified NK cells from control and E2 (1  $\mu$ M) groups for microarray analysis. In the present study, we focused to analyze the gene expression profile related to cell proliferation, cytokines, activating and inhibitory receptors and the down-stream signal transduction of NK cells. The results showed that Stat4, Fyn, Sh2d1a, Eat2, Cd244 (2B4) and Irf1 were down-regulated, Runx1 and Irf7 were not changed, and Irf5, Esrra and Nr5a1 were up-regulated (Table 1).

**Table 1.** E2 treatment alters gene expression profile in purified NK cells using DNA microarray assay

Name	Description	Cy3/Cy5
Stat4	Signal transducer and activator of transcription 4	0.0961
Fyn	Proto-oncogene tyrosine-protein kinase FYN	0.2922
Sh2d1a	SH2 domain protein 1A (SLAM-associated protein, SAP)	0.3352
Eat2	EWS/FLI1 activated transcript 2 (EAT-2)	0.4576
Cd244	Natural killer cell receptor 2B4 precursor	0.4618
Irf1	Interferon regulatory factor 1 (IRF-1)	0.4988
Runx1	Runt-related transcription factor 1	1.1089
Irf7	Interferon regulatory factor 7 (IRF-7)	1.5896
Irf5	Interferon regulatory factor 5 (IRF-5)	2.2174
Esrra	Steroid hormone receptor ERR1 (Estrogen-related receptor- $\alpha$ )	2.0469
Nr5a1	Steroidogenic factor 1 (STF-1) (SF-1)	3.0748

The value of Cy3/Cy5 > 2 or < 0.5 indicated significant up-regulation or down-regulation of the gene expression.

## Discussion

Our *in vitro* experiments showed E2 can suppress NK cell cytotoxicity and proliferative capacity. The evidence accumulated in the past few years has indicated that changes in E2 levels affect both conventional and uterine NK cell subsets. For instance, in pregnant women, NK cell activity declines during the second and third trimesters of pregnancy (17). Animal studies provided direct evidence: chronic administration of high doses of E2 to mice resulted in a decreased number and activity of NK cells (18). Our present results were consistent with these previous reports. But contrary to our findings, Kitaya reported that E2 had no significant effects on the proliferation, cytolytic activity, and cytokine secretion of human endometrial CD16<sup>neg</sup>CD56<sup>bright</sup> NK cells. One possible explanation for this discrepancy is that endometrial CD16<sup>neg</sup>CD56<sup>bright</sup> NK cells are activated in the human endometrium, in contrast to peripheral lymphocytes, which are in a resting state (6).

E2 reduces NK cell cytotoxicity and proliferative capacity possibly through influencing the phenotypes of NK cells. Here we focused on four molecules, CD122, CD94, CD69 and Ly49. CD122, a subunit of IL-2/IL-15 receptor (IL-2R $\beta$ ), was induced to high expression with the treatment of E2. This indicated that CD122 might be responsible for NK cytotoxicity. It was generally thought that CD69 played a role in initiating the cell activation and regulating NK cell functions such as proliferation (14). But in our experiments, the change of CD69 expression was not observed with the exposure of NK cells to E2. The cytotoxicity of NK cells was down-regulated by the negative signal mediated through inhibitory receptors (19). Three inhibitory receptor families are currently known, the killer cell Ig-like receptors (KIR)

found in humans, the Ly49 lectin-like receptors found in mice, and the CD94/NKG2A lectin-like receptors shared by humans and mice (20, 21). Our results showed a significant increase in the expression of CD94 on NK cells. Previous studies showed the increased expression of inhibitory receptors including CD94/NKG2A on peripheral NK cells in the first weeks of pregnancy, reaching a maximum within the third month of gestation with a subsequent decline to basal levels by the end of pregnancy. This suggested that up-regulation of CD94 may lead to low cytolytic activity. Murine NK cells express multiple Ly49 receptors. These receptors either inhibit or activate NK cell functions, including cytolysis and cytokine secretion. All previous *in vitro* studies had demonstrated that most activating/inhibitory pairs were dominated by the negative signal (22). But in our present study, the change of Ly49 expression was not observed with the exposure of NK cells to E2. So it was needed to clarify whether CD69 and Ly49 expressions were regulated by exposure to E2.

The cytokines produced by NK cells, such as IFN- $\gamma$  and TNF- $\alpha$ , affecting their survival or proliferation, in part, cytotoxicity function to modulate host immune responses (23). IFN- $\gamma$  is expressed to induce cytotoxicity in response to different extracellular signals (24), while TNF- $\alpha$  secretion is critical for subsequent processes of NK activation (25). Our results showed that E2 inhibited IFN- $\gamma$  but did not affect TNF- $\alpha$  production from PMA-stimulated NK cells, which may be one of the mechanisms contributed to low cytotoxicity. E2 suppressed the production of IFN- $\gamma$  when mouse splenocytes were stimulated by LPS (26), but E2 did not influence TNF- $\alpha$  secretion of LPS-stimulated monocytes in humans (27). Our results seemed consistent with these previous reports.

In order to detect the possible genes involved in the E2-mediated purified NK cell cytotoxicity and proliferation, the cDNA microarray analysis was performed. The results showed that Stat4, Fyn, Sh2d1a, Eat2, Cd244 and Irf1 were down-regulated, Runx1 and Irf7 were not changed, and Irf5, Esrra and Nr5a1 were up-regulated. Stat4 was identified as a key factor for introduction of IFN- $\gamma$  expression (28). Sh2d1a gene codes SLAM-associated protein (SAP). SAP is involved in critical aspects of 2B4 signaling. 2B4 can mediate inhibitory signals in the absence of SAP. SAP is also involved in the positive signaling by 2B4 through recruiting and activating the Src family kinase Fyn. The regulation of SAP expression is paralleled by the ability of 2B4 to stimulate NK cell cytotoxicity (29). Runx transcription factor can control the expression of NK cell receptors. Runx proteins play important roles in NK cell development and functions through control of the expressions of CD122 and IFN- $\gamma$  (30). IRF-5 can also act as a repressor of IFN- $\alpha/\beta$  expression through binding to IRF-7, which results in a heterodimer unable to bind DNA (31). IRF-1 is an important transcription factor in IFN- $\gamma$ -mediated signaling in the development and function of NK cells. IRF-1 also has direct anti-proliferative effects, thus acting as a tumor suppressor and tumor susceptibility gene (32). Estrogen receptor-related

receptors (ERRs) are orphan nuclear receptors and can bind to estrogen receptor elements and estrogen receptor element-related repeats. ERRs can cross talk with ERs in different cell types *via* competition of DNA binding sites and coactivators. ERRs can also regulate cell growth and modulate ER's signaling pathways in multiple ways (33). It suggests that regulation in these genes may be related to the cytotoxicity, proliferation and cytokine expression of the NK cells mediated by E2. Further research should be conducted to determine the genes modulated by exposure to E2.

In conclusion, our findings pointed out that E2 affects the NK maturation and function. E2 suppresses the NK cell cytotoxicity and proliferative capacity *in vitro*, potentially through influencing the phenotypes and cytokine expression of NK cells involving CD94 and IFN- $\gamma$ . Furthermore, Stat4, Fyn, Sh2d1a, Eat2, Cd244, Irf1, Runx1, Irf7, Irf5, Esrra and Nr5a1 genes might also be involved in the cytotoxicity, proliferation and cytokine expression of E2-mediated purified NK cells. The inhibition of NK cells might explain fetus tolerance during the pregnancy.

## Acknowledgements

This work was supported by the Project Foundation of Jiangsu Province Department of Health (No. H200754) and Special Research Grant for the Key Laboratory from the Department of Health, Jiangsu Province (XK200709 to YH).

## References

- Thellin O, Heinen E. Pregnancy and the immune system: between tolerance and rejection. *Toxicol.* 2003;185:179-184.
- Dosiou C, Giudice LC. Natural killer cells in pregnancy and recurrent pregnancy loss: Endocrine and immunologic perspectives. *Endocrine Rev.* 2005;26:44-62.
- Ashkar AA, Croy BA. Functions of uterine natural killer cells are mediated by interferon  $\gamma$  production during murine pregnancy. *Seminars Immunol.* 2001;13:235-241.
- Koopman LA, Kopcow HD, Rybalov B, et al. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J Exp Med.* 2003;198:1201-1212.
- Van Den Heuvel M, Horrocks J, Basher S, et al. Menstrual cycle hormones induce changes in functional interactions between lymphocytes and decidual vascular endothelial cells. *J Clin Endocrinol Metab.* 2005;90:2835-2842.
- Kitaya K, Yasuda J, Nakayama T, Fushiki S, Honio H. Effect of female sex steroids on human endometrial CD16<sup>neg</sup>CD56<sup>bright</sup> natural killer cells. *Fertil Steril.* 2003;79(Suppl 1):730-734.
- Yang LS, Hu YL, Hou YY. Effects of 17 $\beta$ -estradiol on the maturation, nuclear factor  $\kappa$ B p65 and functions of murine spleen CD11c-positive dendritic cells. *Mol Immunol.* 2006;43:357-366.
- Curran EM, Berghaus LJ, Verneti NJ, Saporita AJ, Lubahn DB, Estes DM. Natural killer cells express estrogen receptor- $\alpha$  and estrogen receptor- $\beta$  and can respond to estrogen *via* a non-estrogen receptor- $\alpha$ -mediated pathway. *Cell Immunol.* 2001;214:12-20.
- Sorachi K, Kumagai S, Sugita M, Yodoi J, Imura H. Enhancing effect of 17 $\beta$ -estradiol on human NK cell activity. *Immunol Lett.* 1993;36:31-35.
- Souza SS, Castro FA, Mendonc HC, et al. Influence of menstrual cycle on NK activity. *J Reprod Immunol.* 2001;50:151-159.
- Hao S, Zhao JL, Zhou JJ, et al. Modulation of 17 $\beta$ -estradiol on the number and cytotoxicity of NK cells *in vivo* related to MCM and activating receptors. *Inter Immunopharmacol.* 2007;7:1765-1775.
- Ferenczi K, Burack L, Pope M, Krueger JG, Austin LM. CD69, HLA-DR and the IL-2R identify persistently activated T cells in psoriasis vulgaris lesional skin: blood and skin comparisons by flow cytometry. *J Autoimmun.* 2000;14:63-78.
- Kim S, Yokoyama WM. NK cell granule exocytosis and cytokine production inhibited by Ly-49A engagement. *Cell Immunol.* 1998;183:106-112.
- Yokoyama WM, Plougastel BF. Immune functions encoded by the natural killer gene complex. *Nat Rev Immunol.* 2003;3:304-316.
- Marcusson-Stahl M, Cederbrant K. A flow-cytometric NK-cytotoxicity assay adapted for use in rat repeated dose toxicity studies. *Toxicol.* 2003;193:269-279.
- Takeda K, Cretney E, Hayakawa Y, et al. TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver. *Blood.* 2005;105:2082-2089.
- Beagley KW, Gockel CM. Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. *FEMS Immunol Med Microbiol.* 2003;38:13-22.
- Yovel G, Shakhar K, Ben-Eliyahu S. The effects of sex, menstrual cycle, and oral contraceptives on the number and activity of natural killer cells. *Gyn Oncol.* 2001;81:254-262.
- Dimasi N, Sawicki MW, Reineck LA, et al. Crystal structure of the Ly49I natural killer cell receptor reveals variability in dimerization mode within the Ly49 family. *J Mol Biol.* 2002;320:573-585.
- Vosshenrich C, Samson-Ville'ger S, Santo J. Distinguishing features of developing natural killer cells. *Cur Opin in Immunol.* 2005;17:151-158.
- Zhang C, Zhang J, Sun R, Feng J, Wei H, Tian Z. Opposing effect of IFN $\gamma$  and IFN $\alpha$  on expression of NKG2 receptors: negative regulation of IFN $\gamma$  on NK cells. *Int Immunopharmacol.* 2005;5:1057-1067.
- Ortaldo JR, Young HA. Mouse Ly49 NK receptors: balancing activation and inhibition. *Mol Immunol.* 2005;42:445-450.
- Raulet DH. Interplay of natural killer cells and their receptors with the adaptive immune response. *Nat Immunol.* 2004;5:996-1002.
- Sakurai T, Wakimoto N, Yamada M, et al. Effect of macrophage colony-stimulating factor on mouse NK1.1<sup>+</sup> cell activity *in vivo*. *Int J Immunopharmacol.* 1998;20:401-413.
- Gan X, Zhang L, Solomon GF, Bonavida B. Mechanism of norepinephrine-mediated inhibition of human NK cytotoxic functions: inhibition of cytokine secretion, target binding, and programming for cytotoxicity. *Brain Behav Immun.* 2002;16:227-246.
- Nakaya M, Yamasaki M, Miyazaki Y, Tachibana H, Yamada K. Estrogenic compounds suppressed interferon- $\gamma$  production in mouse splenocytes through direct cell-cell interaction *in vitro*. *Cell Dev Biol Anim.* 2003;9:383-387.
- Bouman A, Sxhipper M, Heineman MJ, Faas M. 17 $\beta$ -estradiol and progesterone do not influence the production of cytokines from lipopolysaccharide-stimulated monocytes in humans. *Fertil Steril.* 2004;82(Suppl 3):1212-1219.
- Thierfelder WE, Van Deursen JM, Yamamoto K, et al. Requirement for Stat4 in IL-12-mediated responses of NK and T

- cells. *Nature*. 1996;382:171-174.
29. Endt J, Eissmann P, Hoffmann SC, Meinke S, Giese T, Watzl C. Modulation of 2B4 (CD244) activity and regulated SAP expression in human NK cells. *Eur J Immunol*. 2007;37:193-198.
  30. Ohno S, Sato T, Kohu K, et al. Runx proteins are involved in regulation of CD122 and IFN $\gamma$  expression during NK cell differentiation. *Int Immunol*. 2007;20:71-79.
  31. Barnes BJ, Field AE, Pitha-Rowe PM. Virus-induced heterodimer formation between IRF-5 and IRF-7 modulates assembly of the IFN- $\alpha$  enhanceosome *in vivo* and transcriptional activity of IFN- $\alpha$  gene. *J Biol Chem*. 2003;278: 16630-16641.
  32. Liu J, Guan X, Ma X. IRF-1 is an essential and direct transcriptional activator for interferon  $\gamma$ -induced RANTES/CC15 expression in macrophages. *J Biol Chem*. 2005;280:24347-24355.
  33. Cheung CP, Yu S, Wong KB, et al. Expression and functional study of estrogen receptor-related receptors in human prostatic cells and tissues. *J Clin Endocrinol Metab*. 2005;90:1830-1844.