Regular Article

Natural killer cells become tolerogenic after interaction with apoptotic cells

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Abbreviations: AC, apoptotic cell; AC^{JUR}, apoptotic Jurkat T cells; AC^{CD4}, apoptotic $CD4^{+}$ T cell; apoptotic PLB-985 cells, AC^{PLB}; PS, phosphatidylserine.

Abstract

Natural killer (NK) cells are effectors in innate immunity, but also participate in immunoregulation through the release of transforming growth factor (TGF)-β1 and lysis of activated/autoreactive T cells. Apoptotic cells (ACs) have been shown to induce tolerogenic properties of innate immune cells, including macrophages and dendritic cells, but not NK cells. In this study, we demonstrated that after interaction with ACs, NK cells released TGF-β1, which in turn suppressed the production of interferon (IFN)-γ by NK cells upon IL-12 and IgG activation. We further identified phosphatidylserine (PS) as a potential targets on ACs for the NK cells, since PS could stimulate NK cells to release TGF-β1, which in turn suppressed $CD4^+$ T cell proliferation and activation. Moreover, AC-treated NK cells displayed cytotoxicity against autologous activated $CD4^+$ T cells by upregulating NKp46. We then identified that this lysis occurs in part through NKp46-Vimentin pathway, since activated $CD4^+$ T cells expressed vimentin on cell surface and blocking of vimentin or NKp46, but not other NK cell receptors, significantly suppressed the NK cell cytotoxicity. We report here a novel interaction between NK cells and ACs resulting in the tolerogenic properties of NK cells required for immune contraction.

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Introduction

Natural killer (NK) cells are the front line players of innate immunity, involved in eliminating virus infected cells and transformed cells [1]. NK cells also promote inflammation, with release of high levels of pro-inflammatory cytokines and chemokines. However, recent observations revealed that they are important in immune regulation and linking the innate and adaptive immunity [2]. NK cells could regulate immune responses by production of cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-10 [3]. Martin-Fontecha et al. further demonstrated that NK cells can release IFN-γ for Th1 polarization *in vivo* [4]. Moreover, activated NK cells could acquire antigen presentation cells phenotype with upregulation of CD86 and MHC class II molecules to enhance T cell proliferation and activation [5].

Although NK cells participate in promoting inflammation and Th1 polarization, deficient NK cell functions and cytotoxicity are associated with autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis [6,7]. These observations suggest that NK cells help maintain homeostasis of the immune system. They may play a role in peripheral tolerance

as they can produce transforming growth factor (TGF)-β1 [8], which is further enhanced by coculturing with anti-CD2 antibody [9] and soluble HLA-I [10]. *In vivo* mouse model confirmed that TGF-β1 producing NK cells can inhibit Th1 response by suppressing self production of IFN-γ [11]. Furthermore, NK cells display cytolytic activity against autologous immune cells such as DCs and T cells. Lysis of immature DCs by NK cells through NKp30 can limit the number of mature DCs and prevent over presentation of antigens to T cells [12]. NK cells also eliminate autoreactive T cells in experimental autoimmune encephalomyelitis (EAE) mouse model resulting in less disease severity [13,14]. Cerboni et al. demonstrated that IL-2 activated NK cells lyse autologous activated CD4^{+} T cells through NKG2D/NKG2DLs interaction *in vitro* [15].

Apoptotic cells (ACs) are self antigens that induce tolerogenic properties of innate immune cells such as macrophages and DCs which can recognize and phagocytose ACs [16-18]. After ingestion of ACs, macrophages can release TGF-β1 to resolve inflammation *in vivo* of the LPS-stimulated lungs [19]. DCs similarly produce TGF-β1 and IL-10 after ingestion of ACs and acquire the antigen presenting cells (APCs) phenotype with homing capability to lymph

nodes [17,18], inducing T cell tolerance by immunosuppressive cytokine production and/or self antigen presentation [20]. Therefore, ACs can induce immune tolerance when they are recognized by macrophages and DCs under physiologic status. However, the interaction between ACs and NK cells has not been studied.

During inflammation, NK cells are recruited to induce apoptosis of virus infected cells or transformed cells. Previous studies demonstrated that NK cells are inactivated after killing of sensitive target cells [21,22]. We hypothesize these resulting ACs can negatively feedback on NK cells for immune contraction. In this study, we designed our *in vitro* model to reprise the interaction between NK cells and ACs, investigating whether NK cells become tolerogenic.

Results

AC-treated NK cells released TGF-β1 to suppress self production of IFN-γ

More than 95% of Jurkat T cells and autologous CD4$^+$ T cells became Annexin V$^+$ and propidium iodide$^+$ after 50 hr of UV irradiation (Fig. S1). We first investigated the production of TGF-β1 by NK cells after cocultured with apoptotic Jurkat T cells (AC^{Jur}) (Fig. 1A) or apoptotic autologous CD4$^+$ T cells (AC^{CD4}) (Fig. 1B) for 36 hr. Untreated NK cells and ACs alone were used as negative controls and only minimal levels of TGF-β1 were detected (Fig. 1A and B). We observed that both AC^{Jur}- and AC^{CD4}-treated NK cells released significantly higher level of TGF-β1 when compared with untreated NK cells (P< 0.05, n= 5) (Fig. 1A and B). Other cytokines, including IFN-γ and TNF-α, were not detected in any conditions (data not shown).

Since TGF-β1 could down-regulate NK cell function in terms of IFN-γ production [11, 23], we hypothesized that ACs may inhibit IFN-γ production from NK cells by promoting NK cells to release TGF-β1. We first demonstrated that AC^{Jur}-treated NK cells were less responsive to IL-12 in producing IFN-γ (Fig. 1C). To further delineate the mechanism of this phenomenon, we blocked the

function of TGF-β1 by adding TGF-β1 neutralization antibody to the culture. We observed the inhibition of IFN-γ on IL-12 response was partially reversed (Fig. 1C). We also prepared IgG-opsonized AC^{JUR} (Fig S2) to activate NK cells through Fcγ receptors to release IFN-γ (Fig.1D). Similar to IL-12 activation, the addition of TGF-β1 neutralization antibody significantly enhanced the IFN-γ production by NK cells. Thus, TGF-β1 was partially responsible for the suppression of IFN-γ production from NK cells observed in our experiments. AC^{JUR} alone did not have IFN-γ production (data not shown).

NK cells released TGF-β1 after interaction with Phosphatidylserine (PS)

PS is exposed on cell surface during apoptosis [24] and the interaction of ACs with phagocytes through PS resulted in the production of TGF-β1 [19]. Therefore, we have used apoptotic PLB-985 cells (AC^{PLB}), which do not expose PS on the cell surface during apoptosis [19], to investigate the role of PS on ACs to induce TGF-β1 production from NK cells. We observed that AC^{PLB} was not able to induce TGF-β1 production from NK cells (Fig 2A). Then, we investigated whether PS alone could stimulate NK cells to release TGF-β1. After cocultured with PS, NK cells released TGF-β1 in a PS dose-dependent manner (Fig. 2B) and

the percentage of TGF-β1$^+$ NK cells was also increased (Fig. 2C). We have also screened for the expression of several PS receptors, i.e. CD36, Tim-1 and Tim-4, in resting, IL-2 activated, AC^{JUR}- and PS-treated NK cells by flow cytometry, but none of them were detected (data not shown).

PS-treated NK cells inhibited the proliferation and activation of autologous CD4$^+$ T cells

We next investigated the regulatory role of these PS-treated NK cells on the proliferation and activation of autologous CD4$^+$ T cells. CD4$^+$ T cells were activated with immobilized anti-CD3 antibody. After adding of NK cells that were pre-treated with 50μg of PS, the percentage of dividing activated CD4$^+$ T cells was decreased from ~80% to ~40% as determined by flow cytometric CFSE assays (Fig. 3A). Also, 50μg PS-treated NK cells suppressed the expression of CD25, a T cell activation marker, of activated CD4$^+$ T cells from ~85% to ~30% (Fig. 3B).

Since PS-treated NK cells released TGF-β1, which could inhibit T cell proliferation and activation [25], we added TGF-β1 neutralization antibody to

investigate whether TGF-β1 was involved in the suppression of the CD4$^+$ T cells

observed in our system. The addition of the antibody partially restored the

proliferation (Fig. 4A) and CD25 expression (Fig. 4B) of CD4$^+$ T cells. Therefore,

TGF-β1 was involved in the inhibitory effect of PS-treated NK cells on CD4$^+$ T

cells.

To further investigate the mechanism of T cells suppression by PS-treated NK

cells, we have investigated the cytotoxicity of PS-treated NK cells against

anti-CD3-activated autologous CD4$^+$ T cells. However, the cytotoxicity was not

increased (data not shown). Thus, the suppression of activated T cells by

PS-treated NK cells is independent of direct cell lysis.

AC^{JUR}-treated NK cells displayed higher cytotoxicity against autologous activated CD4$^+$ T cell

Previous studies have highlighted the potential role of NK cells in T cell

surveillance by cytotoxicity [26]. We hypothesized that NK cells may suppress

CD4$^+$ T cells through their cytolytic activities after cocultured with ACs.

Therefore, we cocultured AC^{JUR}-treated NK cells with autologous PHA activated

CD4$^+$ T cells and the cytotoxicity was determined by both cytoloytic and CD107a assays. As shown in Fig. 5A, AC^{JUR}-treated NK cells displayed higher cytotoxicity against autologous PHA activated CD4$^+$ T cells at NK cells to T cells ratio of 50:1 when compared with untreated NK cells (P< 0.05, n= 6, Fig. 5A). Similar phenomenon was observed when using autologous PMA/Ionomycin activated CD4$^+$ T cells (data not shown). For autologous resting CD4$^+$ T cells, both AC^{JUR}-treated and untreated NK cells displayed minimal cytotoxicity (Fig. 5A). Consistent with the cytolytic assay, AC^{JUR}-treated NK cells expressed higher CD107a, a marker of degranulation in NK cells, when they were cocultured with activated autologous CD4$^+$ T cells (Fig. 5B). We have performed the control experiments to coculture activated CD4$^+$ T cells with ACs alone and there was no significant increase of T cell death when compared with activated CD4$^+$ T cells alone.

AC^{JUR}-treated NK cells mediated lysis of autologous activated CD4$^+$ T cell through NKp46 pathway

To determine the NK cell receptor(s) responsible for direct killing of autologous activated CD4$^+$ T cells, we determined the surface expressions of the three natural

cytotoxicity receptors (NCRs), i.e. NKp30, NKp44, NKp46, and also NKG2D on

AC^{JUR}-treated NK cells (Fig. 6A). We observed that NKp46 was significantly

upregulated on AC^{JUR}-treated NK cells (MFI: 18.76 ± 2.01), when compared with

untreated NK cells (MFI: 13.07 ± 1.41) ($P < 0.05$; n= 6) (Fig. 6B).

To confirm the role of NKp46 in autologous activated CD4$^+$ T cell killing, we

blocked NKp46 on AC-treated NK cells by using NKp46 blocking antibody. After

blocking, the cytotoxicity of AC^{JUR}-treated NK cells against autologous PHA

activated CD4$^+$ T cells was significantly reduced when compared with the isotype

control ($P < 0.05$, n= 4) (Fig. 6C). Therefore NKp46 was involved in the killing of

autologous activated CD4$^+$ T cells by AC^{JUR}-treated NK cells. We have also

investigated the role of ICAM-1, 2B4 and TGF-β1 in this killing by using

corresponding neutralization antibodies, however, no significant change was

observed (data not shown).

To investigate the role of PS in upregulating NKp46 expression and cytotoxicity

of AC-treated NK cells, we incubated NK cells with AC^{JUR} or AC^{PLB} and

observed that NKp46 levels were significantly upregulated in both treatments with

comparable levels (Fig S3A). Moreover, PS-treated NK cells did not show increase cytotoxicity against autologous PHA-activated CD4^{+} T cells (Fig S3B). Thus, the increased cytotoxicity of AC-treated NK cells was independent to PS on ACs.

NK cells killed activated CD4^{+} T cells through NKp46-vimentin pathway

Nolte-'t Hoen et al. reported that NK cells recognize and bind mitotic T cells through NKp46 [27], with our own observation, we hypothesized that activated T cells may express NKp46 ligand(s) through which NK cell cytotoxicity is mediated. Vimentin, an intermediate filament protein expressed on the surface of Sezary cells [28], TNF-α activated macrophages [29] and *Mycobacterium tuberculosis*-infected monocytes [30], has been reported as a NKp46 ligand that can induce the cytolytic activity of NK cells [30]. Therefore, we investigated the surface expression of vimentin on activated CD4^{+} T cells and its role in mediating NK cell cytotoxicity.

We observed that the percentages of surface vimentin expressing cells in PHA (13.1 ± 2.2%) and PMA/Ionomycin (24.9 ± 1.9%) activated CD4^{+} T cells were

significantly higher than in resting CD4$^+$ T cells (7.0 ± 2.9%) with P< 0.05 and

0.005 respectively (n= 4) (Fig. 7A). In concordance with Garg et al.(29), we

confirmed that vimentin is a NKp46 ligand as anti-vimentin antibody could block

the binding of NKp46-Ig fusion protein on PHA activated CD4$^+$ T cells (Fig. 7B).

Then, we demonstrated that the cytotoxicity of AC^{JUR}-treated NK cells against

PHA-activated CD4$^+$ T cells was significantly reduced after pre-incubation of

these T cells with anti-vimentin antibody (P< 0.005, n= 4) (Fig. 7C). However,

double blocking of NKp46 and vimentin by using neutralization antibodies did

not further suppress the cytotoxicity (Fig. 7C). Therefore, we concluded that

vimentin is one of the ligands that could be recognized by NKp46 and this

recognition leads to killing of activated CD4$^+$ T cell by NK cells.

NK cells did not phagocytose ACs

ACs are usually phagocytosed by macrophages and DCs through the recognition

of PS [16-18]. It is possible that NK cells may also phagocytose ACs as we have

demonstrated that NK cells could interact with both ACs and PS. However,

confocal microscopy analysis showed that NK cells did not uptake AC^{JUR} up to

48hr (data not shown).

Discussion

In this study, we demonstrated a novel interaction between NK cells and ACs resulting in NK cells acquiring tolerogenic properties mediated by release of TGF-β1 in suppressing the production of IFN-γ by NK cells. This interaction involve recognition by NK cells of PS on the ACs, since PS-treated NK cells also released TGF-β1 resulting in inhibition of CD4$^+$ T cell proliferation and activation. We further identified after interaction with ACs, these NK cells killed activated CD4$^+$ T cells by recognizing T cell surface vimentin via its increased expression of NKp46.

In vivo studies confirmed NK cells can trigger Th1 response as they are the initial source of IFN-γ (4), while TGF-β1 producing NK cells suppress Th1 response by inhibiting self production of IFN-γ [11]. In this study, we demonstrated that NK cells released TGF-β1 to suppress their own IFN-γ production after interaction with ACs (Fig. 1C and D). This is an important negative feedback mechanism initiated by NK cells for the normal termination of inflammation and Th1 immune responses. We also observed that TGF-β1 only partially responsible for the suppression of IFN-γ (Fig. 1C). Factors other than TGF-β1 are therefore involved

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in this inhibition. As reported by Fadok et al., macrophages after having ingested ACs release platelet-activating factor (PAF) to inhibit the production of pro-inflammatory cytokines [16]. Since NK cells also produce PAF [31], future studies are required to identify other immunosuppressive factors including PAF that can be released by AC-treated NK cells to inhibit self production of IFN-γ.

PS is exposed on the outer leaflet of plasma membrane during apoptosis and is the ligand recognized on ACs by phagocytes to produce TGF-β1 [19, 26, 32, 33]. NK cells express a wide range of receptors for pattern recognition, including toll like receptors, c-type lectins and scavenger receptors [34, 35], which can participate in recognition of ACs and PS. We demonstrated that PS is also the ligand with which NK cells interact to produce TGF-β1 and suppress the activation and proliferation of CD4$^+$ T cells. We have therefore screened for the expression of several known PS receptors, i.e. CD36, Tim-1 and Tim-4, in resting, IL-2 activated, AC- and PS-treated NK cells by flow cytometry. However, no known tested PS receptors were found (data not shown). Further studies are required to identify the possible PS receptor on NK cells.

Recognition of PS on ACs by phagocytes always leads to two events: (1) the engulfment of ACs by phagocytes, which is initiated by the "eat me" signal [32]; (2) production of immunosuppressive cytokines, including TGF-β1 and IL-10 [16-19,33]. However, we only observed the production of TGF-β1 from AC- or PS-treated NK cells but not the uptake of ACs by NK cells. This indicated that these two events could occur independently and they are not necessarily associated.

Surprisingly, AC-treated NK cells are more potent in killing autologous activated CD4^{+} T cells (Fig. 5) although they release TGF-β1 that is known to suppress NK cell cytotoxicity [20]. It becomes understandable when we observed that only NKp46 was upregulated in AC-treated NK cells (Fig 5A), as TGF-β1 can only suppress NK cell cytotoxicity through down-regulation of NKp30 and NKG2D but not NKp44 and NKp46 [36]. We further identified vimentin expressed on activated CD4^{+} T cell surface as a ligand for NKp46 to induce NK cell cytotoxicity (Fig. 6 and 7). Killing of activated T cells by NK cells is critical in terminating immune responses, as highlighted in familial hemophagocytic lymphohistiocytosis, a form of primary immunodeficiency associated with

defective NK cell cytotoxicity that leads to accumulation and infiltration of activated T cells and macrophages after infection [37]. We proposed that at the late phase of inflammation, NK cells encounter a lot of ACs, resulting in upregulation of NKp46, and lysis of activated T cell through the NKp46-vimentin pathway, and are the regulatory cells for immune contraction.

Although NKG2D-NKG2DLs pathway has been shown to be responsible for IL-2 activated NK cells regulation of the activatied CD4$^+$ T cell survival [15], the expression levels of NKG2D of AC-treated NK cells in our study were similar to untreated NK cells; therefore, it is unlikely that NKG2D is responsible for the differential killing of autologous PHA activated CD4$^+$ T cells. Therefore NK cells lyse activated or autoreactive T cells through different receptors, dependent on the initial stimulation.

In conclusion, we identified a novel interaction that ACs can induce tolerogenic properties of NK cells, which are mediated through production of TGF-β1 and increase of cytotoxicity against activated CD4$^+$ T cells. Defective NK cell functions in autoimmune diseases, such as systemic lupus erythematosus,

rheumatoid arthritis and multiple sclerosis; and primary immunodeficiences, such

as familial hemophagocytic lymphohistiocytosis, have already highlighted the

critical role of NK cells in the termination of immune responses. Future *in-vivo*

studies that focus on NK cells in immune contraction should be performed.

Materials and Methods

AC induction

Jurkat T cells, PLB-985 cells and purified CD4^{+} cells were used as the source of ACs. As described in our previous studies [18], apoptosis was induced by UV irradiation (80 mJ/cm^2) in a Spectrolinker UV Crosslinkers (Spectroline, NY, US). Irradiated cells were cultured in RPMI 1640 medium (Gibco, CA, US) with 0.4% BSA (Sigma-Aldrich, St. Louis, MO) at 37°C in 5% CO_2 to undergo apoptosis for 50 hours. Apoptosis was evaluated by apoptosis detection kit (Immunotech, Marseilles, France) by flow cytometry. Cells were stained by Annexin V[FITC] and propidium iodide according to the manufacturer's protocol. ACs were incubated with purified human IgG (EMD BioSciences, Inc. La Jolla, CA) at 1 mg/ml for 1 hr. After washed twice by PBS, the secondary antibody, FITC conjugated goat anti-human IgG antibody, was added for an additional 30 min for elevating the opsonization of human IgG by flow cytometry.

Cell isolation

Peripheral blood mononuclear cells (PBMCs) from peripheral blood of healthy blood donors were isolated by standard sedimentation over Ficoll-Hypaque

(Amersham Pharmacia Biotech, Piscataway, NJ). This study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of the University of Hong Kong / Hospital Authority Hong Kong West Cluster. NK cells were negatively selected from PBMC by NK Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated population was >94% CD56$^+$ CD3$^-$ as determined by flow cytometry. Contamination of CD3$^+$, CD14$^+$ and CD19$^+$ was <2%. CD4$^+$ cells were positively selected by anti-CD4 immunomagnetic beads with purity consistently >97%.

NK cell culture

Isolated NK cells were cultured with RPMI 1640 medium containing 1% BSA at 37°C in 5% CO_2 at 24-well plates at a concentration of 5 x 10^5 cell/ml. ACs were added to the NK culture for 36 hr at NK cells to ACs ratio of 1:5. In some cases, NK cells were cocultured with ACs with reagents (2 ng/ml of IL-12, 10 µg/ml of anti-TGF-ß1 antibody or isotype control antibody).

Cytokine detection

The cytokines in the supernatant from the coculture of NK cells and ACs were

detected by DuoSet ELISA Development System (R&D Systems, Minneapolis, MN).

PS treatment

PS in chloroform (Avanti Polar Lipids, Alabaster, AL) was diluted by ethanol and dried in 96 well plate. 2×10^5 of NK cells were added to the well with 1% BSA and 30 IU/ml of IL-2 in RPMI 1640 medium for 24 hours.

Intracellular cytokine staining

PS-treated NK cells (2×10^5 per well) were incubated with 10 μg/ml Brefeldin A. After 5 hours, cells were stained with antibodies to surface CD56. Fixation and permeabilization were carried out by using intracellular staining kit (BD Bioscinences, San Diego, CA) and intracellular anti-TGF-β1 antibody (IQ products, Groningen, The Netherlands).

T cell proliferation assays

CD4$^+$ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) before activation. 5×10^4 of these labeled cells were cultured with

different numbers of the PS-treated NK cells at indicated ratios for 5 days with immobilized anti-CD3 antibody. Fcγ receptor blocking reagent (Miltenyi Biotec) was added to block the Fcγ receptor of NK cells. Cell division accompanied by CFSE dilution was analyzed by flow cytometry. CD4$^+$ T cell proliferation was also analyzed by [^{3}H] thymidine incorporation assay as described previously [38,39].

Cytolytic and CD107a assays

The cytolytic activities of NK cells after cocultured with AC^{JUR} were determined by Live/Dead cell-mediated cytotxicity kit (Molecular Probes, Eugene, Ore) and the expression of CD107a on NK cell surface. Autologous CD4$^+$ T cells were activated by PHA (5μg/ml) for 2 days or PMA/Ionomycin (10ng/100ng respectively) for 1 day. After the activation, they were seeded in 96-well plates as target cells and NK cells were added as effector cells at the indicated effector/target cell ratio.

Blocking experiment

Blocking experiments were performed by pre-incubating AC^{JUR}-treated NK cells

with saturating amounts of each of the 3 NCRs and NKG2D blocking antibodies (10-20 μg/ml) for 30 min. PHA activated CD4$^+$ T cells were pre-incubated with anti-vimentin antibody (20 μg/ml) for 30 min. Corresponding isotype antibody was added as control. 5 x 10^5 AC^{JUR}-treated NK cells were cocultured with 1 x 10^4 PHA activated autologous CD4$^+$ T cells for 2 hr for the cytolytic assay.

Antibodies

Mouse antibodies directly conjugated with FITC, PE or PE-Cy5.5 were used (clone): CD3 (UCHT1), CD4 (13B8.2), CD14 (RM052), CD19 (HIB19), CD56 (NCAM16.2), CD107a (H4A3), NKG2D (1D11), NKp30 (30-15), NKp44 (P44-8.1), NKp46 (9E2). They were purchased from BD and Beckman Coulter (Miami, FL). Purified mouse antibodies for blocking experiments were: TGF-β1 (141322), NKp30 (P30-15), NKp44 (P44-8), NKp46 (9E2) and NKG2D (1D11) purchased from Biolegend (San Diego, CA), Beckman Coulter and R&D Systems. Goat anti-human IgG (H+L) and Rabbit anti-goat IgG (H+L) antibodies conjugated with FITC were purchased from Invitrogen (Carlsbad, CA). Goat anti-human vimentin polyclonal antibody was purchased from R&D Systems.

Statistical analysis

Graphs and statistical analyses were performed with the use of Prism5.00 for Windows software (GraphPad Software, San Diego, CA). Pair t-test was used for two columns comparison and ANOVA was used when more than two columns are shown. P value of 0.05 or less was considered significant.

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Conflict-of-interest

The authors have no financial conflict of interest.

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Figure 1.

AC-treated NK cells released TGF-β1 to inhibit self production of IFN-γ.
(A,B) NK cells were incubated with (A) AC^{JUR} or (B) AC^{CD4} for 36 hr.
Supernatants were collected for the detection of total TGF-β1 by ELISA.
Untreated NK cells and ACs alone were used as negative controls *P < 0.05,
paired t-test. (C) NK cells were incubated with IL-12 (2ng/ml) and/or AC^{JUR} in
the presence/absence of anti-TGF-β1 neutralizing antibody (10μg/ml) or an
isotype control. Supernatants were collected for IFN-γ detection by ELISA. *P<
0.05, ANOVA. (D) NK cells were incubated with AC^{JUR} or IgG-opsonized AC^{JUR}
for 36 hr in the presence/absence of TGF-β1 neutralizing antibody (10μg/ml) or
an isotype control and IFN-γ production measured by ELISA. *P<0.05, ANOVA.
Data are displayed as the mean cytokine production ± SEM for at least 4
independent experiments. ND: Not detected.

Figure 2.

The interaction of NK cells with PS induces TGF-β1 production. (A) NK cells
were cocultured with AC^{JUR} or AC^{PLB} or cultured alone for 36 hr and TGF-β1
production measured by ELISA. (B,C) NK cells were cultured with 30 IU/ml of
IL-2 in 96-well plates coated with PS at the indicated concentrations for 24 hr. (B)
TGF-β1 production was determined by ELISA after 24hr. (C) Intracellular
staining using PE conjugated anti-TGF-β1 antibody. Representative flow
cytometry data are shown in the left and middle panels. Combined data (n=4) in
the right panel. Data are mean ± SEM for 4 independent experiments. * P < 0.05,
**P < 0.01, by (A, B) ANOVA and (C) paired t-test.

Figure 3.

PS-treated NK cells inhibit proliferation and activation of CD4$^+$ T cells. (A,B) 5 x 10^4 CFSE-labeled autologous CD4$^+$ T cells were left unstimulated or stimulated with plate-bound anti-CD3 and 2.5 x 10^4 NK cells (untreated or treated with 25 or 50μg PS) were added. (A) The intensities of CFSE were determined by flow cytometry at day 5 (B) APC-conjugated anti-CD25 antibody was used to determine surface expression of CD25 on the CD4$^+$ cells. Grey histogram: isotype control; black line: CD25. The data are representative of 3 independent experiments.

Figure 4.

PS-treated NK cells inhibit proliferation and activation of CD4$^+$ T cells through TGF-β1. (A,B) 5 x 10^4 CFSE-labeled autologous CD4$^+$ T cells were stimulated with plate-bound anti-CD3 and PS-treated NK cells (50 μg PS) were added according to the indicated ratios. 10 μg/ml of TGF-β1 neutralizing antibody or an isotype control antibody were added to the culture. (A) [3H]-Thymidine incorporation was determined after 3 days. (B) Surface expression of CD25 was determined by flow cytometry at day 3. Data are displayed as the mean ± SEM for 3 independent experiments. * P < 0.05, paired t-test.

Figure 5.

AC^{JUR}-treated NK cells mediate lysis of autologous activated CD4$^+$ T cells. (A) NK cells were cocultured with AC^{JUR} for 36 hr and then were separated from

AC^{JUR} by ficoll and cocultured at the indicated ratios with 1 x 10^4 resting or PHA-activated autologous CD4$^+$ T cells for 2 hr. The cytotoxicity of the NK cells is shown as the percentage of PI$^+$ CD4$^+$ T cells in total CD4$^+$ T cells. Data are mean±SEM, n= 6, *p< 0.05, ANOVA. (B) 2 x 10^5 AC^{JUR}-treated NK cells were cocultured with 4 x 10^5 PHA activated autologous CD4$^+$ T cells for 2 hr. The percentage of CD107a$^+$ NK cells after AC^{JUR} treatment was determined by flow cytometry. The data are representative of 4 independent experiments. (C) Combined data for the expression of CD107a on NK and AC^{JUR}-treated NK cells cultured with PHA-activated autologous CD4$^+$ T cells at the indicated ratios (n= 4). *P < 0.05, ANOVA.

Figure 6.

AC^{JUR}-treated NK cells mediate lysis of autologous activated CD4$^+$ T cells through NKp46. NK cells were cocultured with apoptotic Jurkat T cells for 36 hr. (A) The surface expression of NKp30, NKp44 and NKp46, and NKG2D of NK cells were investigated. Results shown are representative of 5 independent experiments. (B) Expression levels of NKp46 (MFI) as determined by flow cytometry. (C) 5 x 10^5 AC^{JUR}-treated NK cells were pre-incubated with saturating amounts of each of the indicated blocking antibodies (10-20 μg/ml) for 30 min. 1 x 10^4 PHA-activated autologous CD4$^+$ T cells were then added and cytotoxicties relative to isotype control were determined. Data are displayed as the mean ± SEM of at least of 4 independent experiments. * P < 0.05 by (B) paired t-test and (C) ANOVA.

Figure 7.

NKp46 recognizes activated autologous CD4$^+$ T cells through vimentin. (A) The percentages of vimentin-expressing cells in resting, PHA-activated and PMA/Ionomycin-activated CD4$^+$ T cells were determined by flow cytometry using a goat anti-human vimentin Ab and an FITC-conjugated secondary rabbit anti-goat IgG antibody. (B) PHA-activated CD4$^+$ T cells were incubated with or without anti-vimentin (20μg/ml) before addition of NKp46-Ig fusion protein (25μg/ml). Flow cytometry using FITC-conjugated secondary anti-human IgG antibody was used to determine NKp46-Ig fusion protein binding. Results are representative of 3 independent experiments. (C) 1 x 10^4 PHA-activated autologous CD4$^+$ T cells were pre-incubated with anti-vimentin antibody, added to 5 x 10^5 AC^{JUR}-treated NK cells that were either untreated or had been pre-incubated with anti-NKp46 for 30 minutes. Cytotoxicties relative to isotype control were then determined. Data are showed as the mean ± SEM for 4 independent experiments. * P < 0.05; ** P < 0.005, ANOVA.

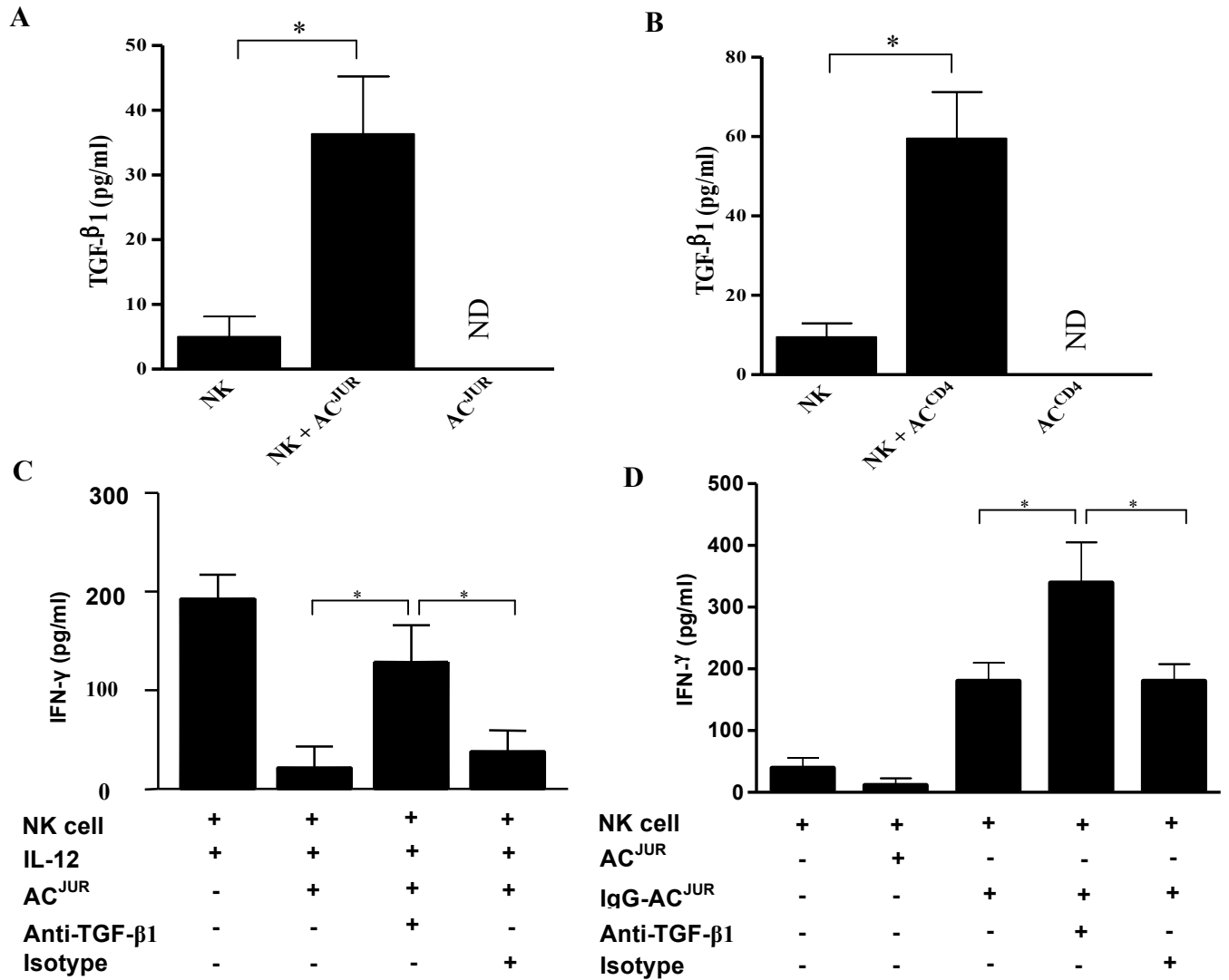**Figure 1. AC-treated NK cells released TGF-β1 to inhibit self production of IFN-γ.**

A

TGF-β1 (pg/ml)

NK — NK + AC^{JUR} — AC^{JUR} (ND)

B

TGF-β1 (pg/ml)

NK — NK + AC^{CD4} — AC^{CD4} (ND)

C

IFN-γ (pg/ml)

NK cell	+	+	+	+
IL-12	+	+	+	+
AC^{JUR}	-	+	+	+
Anti-TGF-β1	-	-	+	-
Isotype	-	-	-	+

D

IFN-γ (pg/ml)

NK cell	+	+	+	+	+
AC^{JUR}	-	+	-	-	-
IgG-AC^{JUR}	-	-	+	+	+
Anti-TGF-β1	-	-	-	+	-
Isotype	-	-	-	-	+

38

Figure 2. The interaction of NK cells with PS induces TGF-β1 production.

Figure 3. PS-treated NK cells inhibit proliferation and activation of CD4$^+$ T cells.

A

untreated CD4$^+$ +anti-CD3

4.5%   88.5%

% of Max

+anti-CD3, +untreated NK   +anti-CD3, +25μg PS-treated NK   +anti-CD3, +50μg PS-treated NK

87.1%   78.8%   45.5%

% of Max

CFSE

B

untreated CD4$^+$ +anti-CD3

4.2%   88.3 %

% of Max

+anti-CD3, +untreated NK   +anti-CD3, +25μg PS-treated NK   +anti-CD3, +50μg PS-treated NK

87.7 %   79.4 %   29.9 %

% of Max

CD25
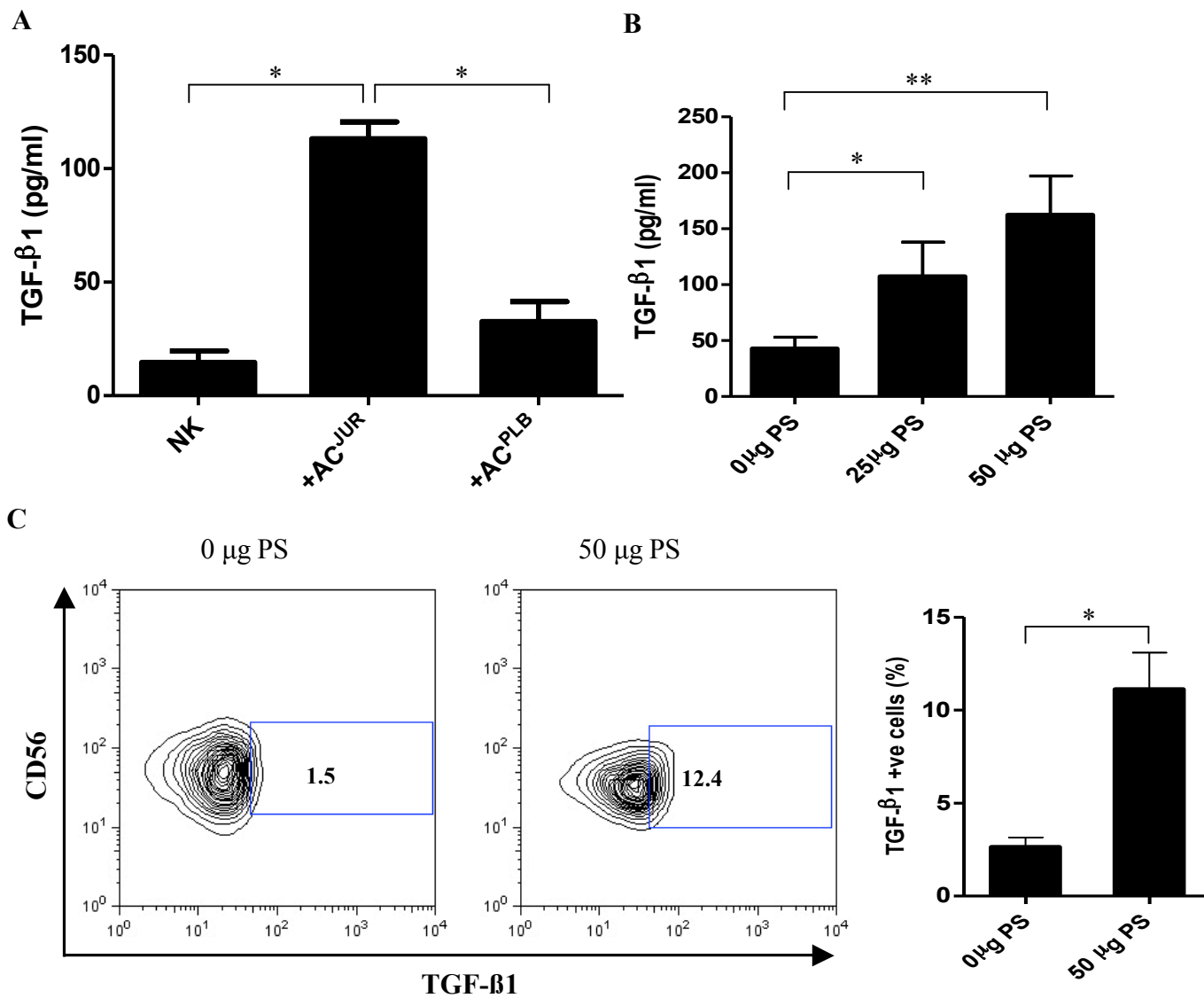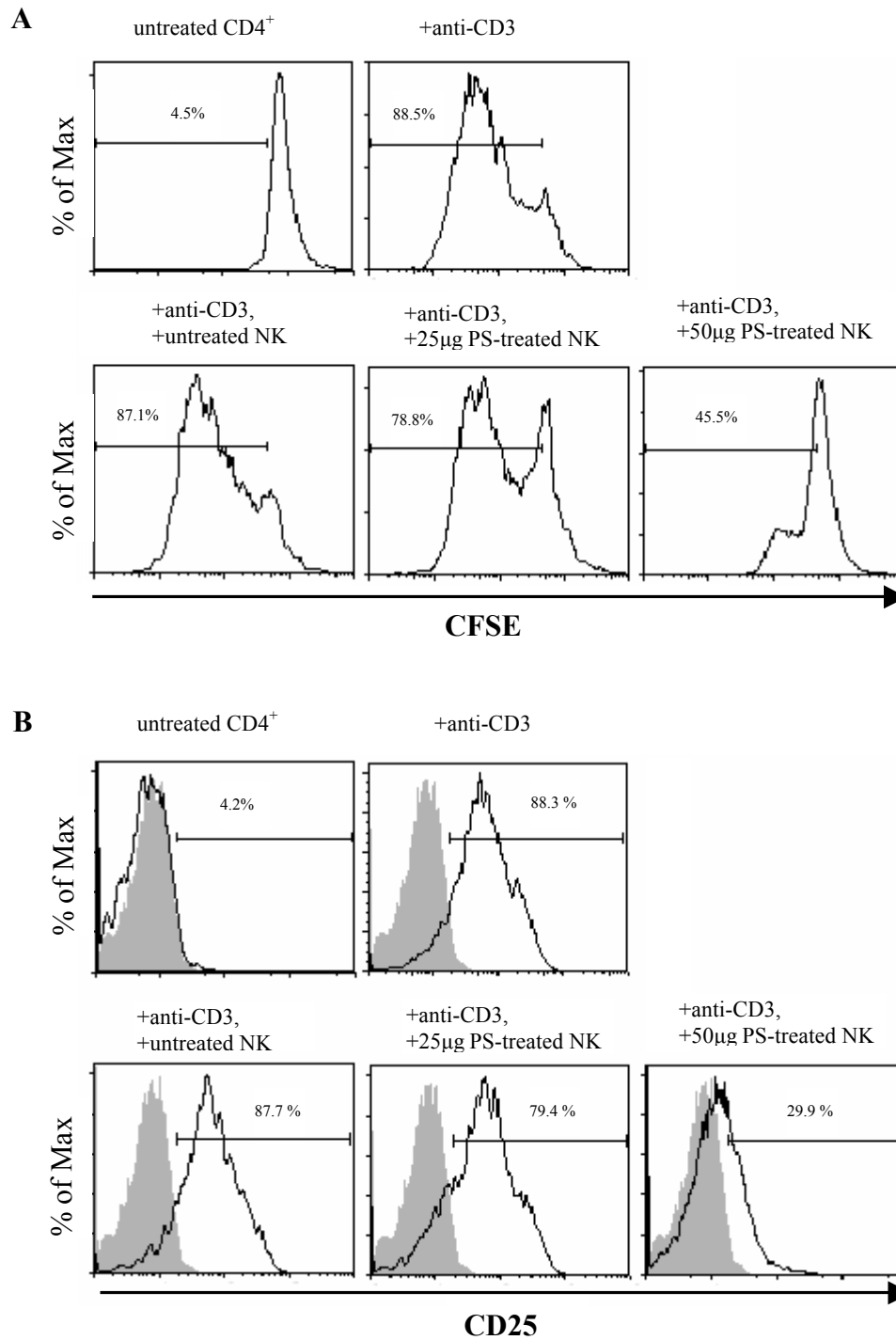
Figure 4. PS-treated NK cells inhibit proliferation and activation of CD4$^+$ cells through TGF-β1.
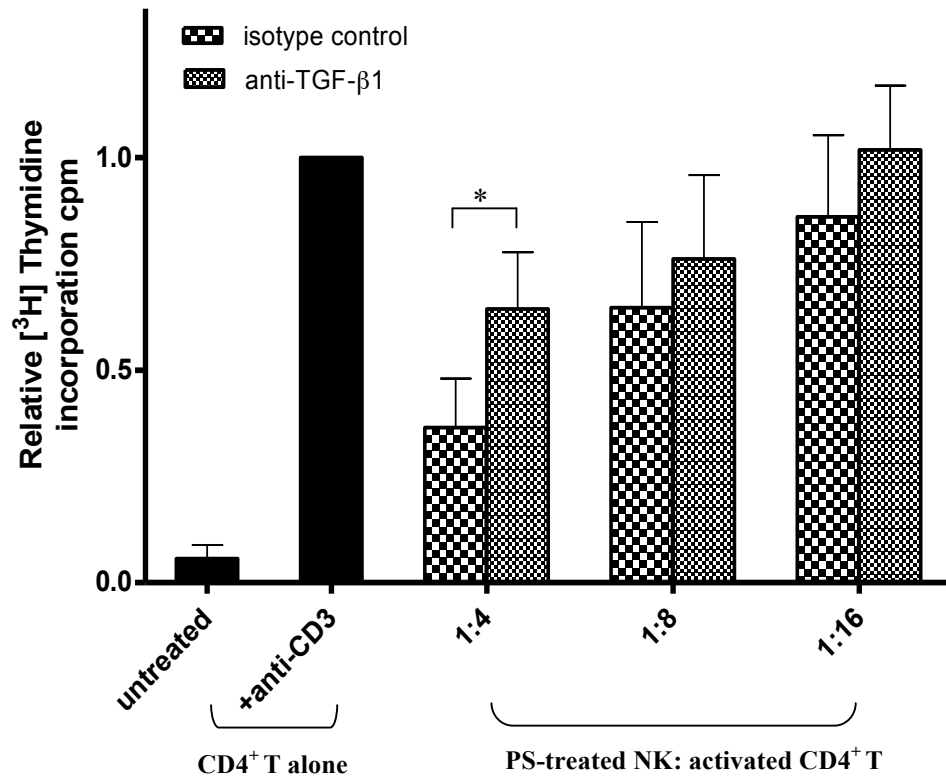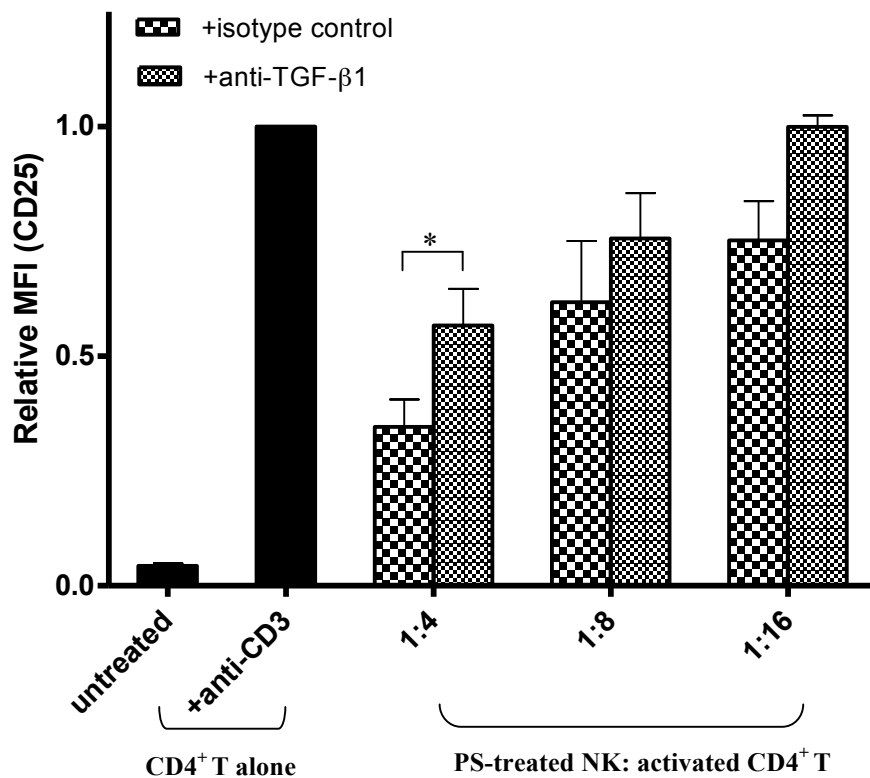
A

B

Figure 5. AC^JUR^-treated NK cells mediate lysis of autologous activated CD4^+^ T cells.
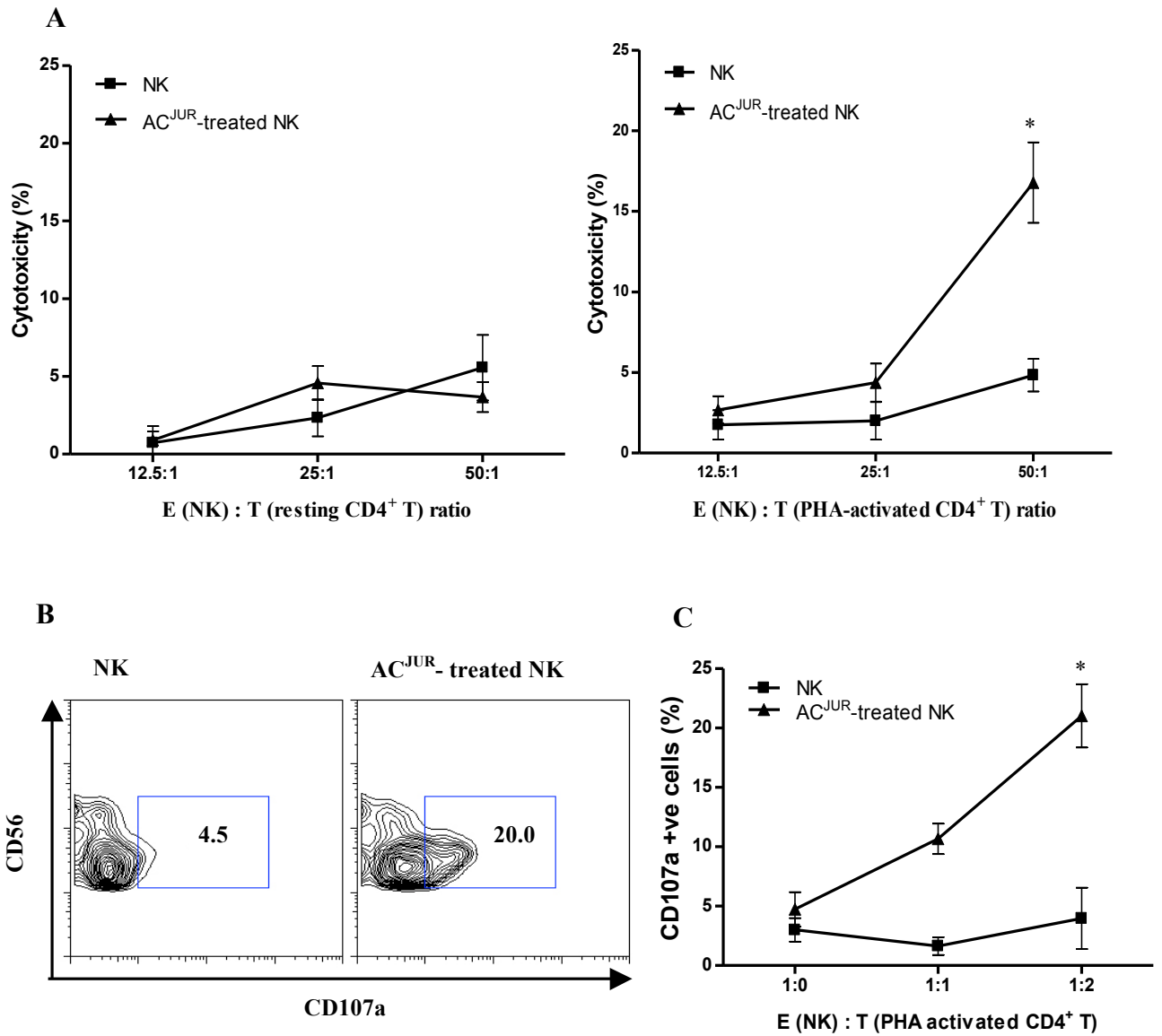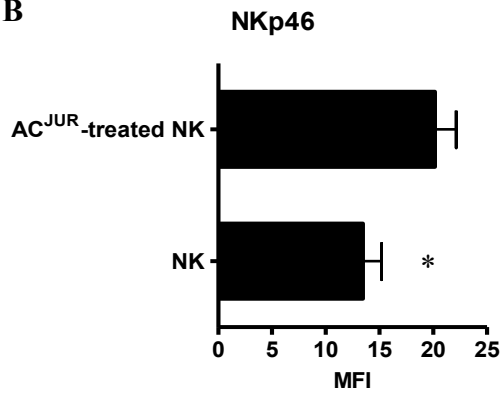
A

B

C

Figure 6. AC^JUR^-treated NK cells mediate lysis of autologous activated CD4$^+$ T cells through NKp46.

A
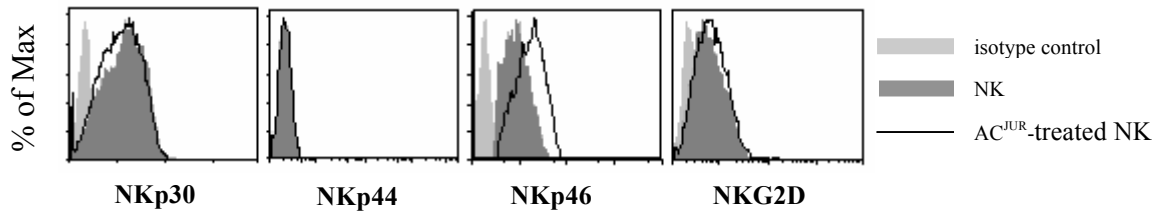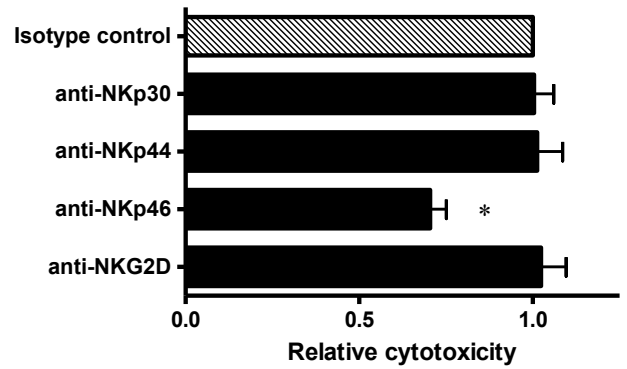
isotype control
NK
AC^JUR^-treated NK

NKp30   NKp44   NKp46   NKG2D

B

NKp46

AC^JUR^-treated NK

NK

*

0   5   10   15   20   25
MFI

C

Isotype control

anti-NKp30

anti-NKp44

anti-NKp46   *

anti-NKG2D

0.0   0.5   1.0
Relative cytotoxicity

Figure 7. NKp46 recognizes activated autologous CD4$^+$ T cells through vimentin

A

B PHA-activated

CD4$^+$ T

NKp46-Ig

+NKp46-Ig +isotype control

+NKp46-Ig +anti-human IgG

+anti-vimentin (pre-treated)
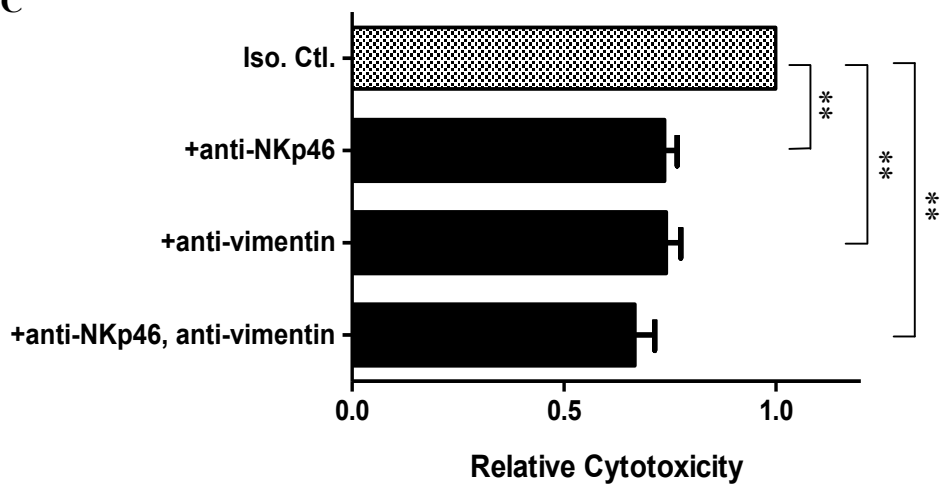+NKp46-Ig +anti-human IgG

C

Relative Cytotoxicity
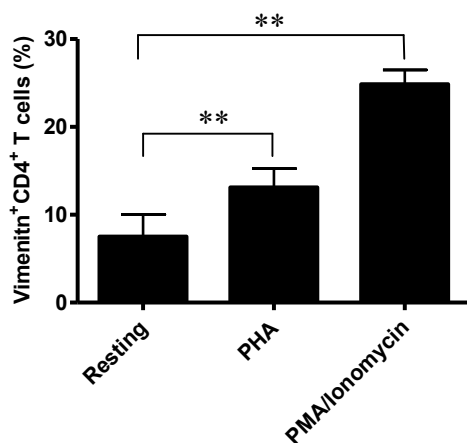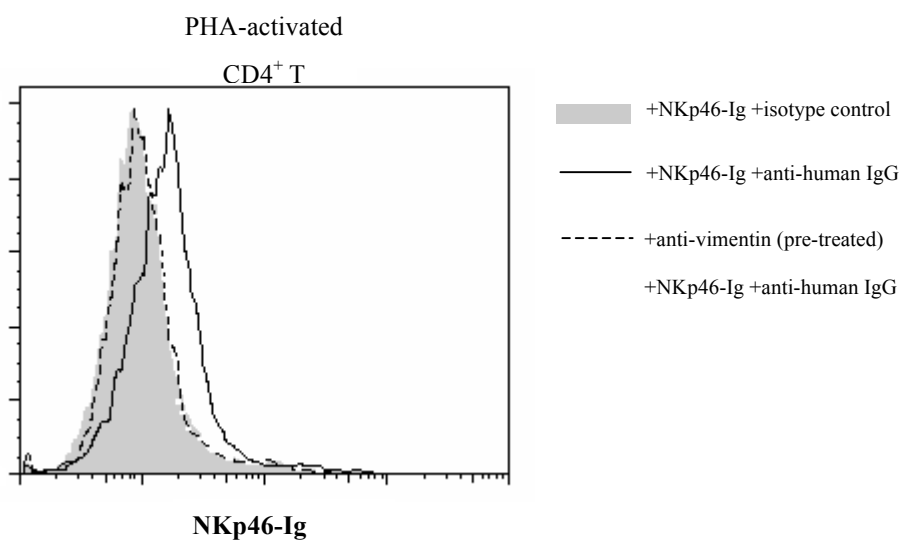
Figure S1.

Induction of apoptotic cells by UV irradiation. Live Jurkat T cells and primary CD4$^+$ T cells were induced to undergo apoptosis by UV irradiation as described in *Materials and Methods*. After 50 hr of UV irradiation, more than 95% of Jurkat T cells and primary CD4$^+$ T cells became annexin V and propidium iodide double positive. Representative of 3 independent experiments. AxV: annexin V; PI: propidium iodide.

Figure S2.

IgG opsonized AC^{JUR} but not live Jurkat T cell. The cells were incubated with or without purified human IgG (1mg/ml) for 1 hr and washed by PBS. A FITC conjugated secondary anti-human IgG antibody was used to elevate the binding (thin line, cells incubate with human IgG; thick line, cells incubate without human IgG). Δ represented the change in mean fluorescence level between the cells with secondary antibody alone versus cells with human IgG followed by secondary antibody. Representative of 3 independent experiments.

Figure S3.

Upregulation of NKp46 and cytotoxicity of AC-treated NK cells were independent to PS. (A) NK cells were incubated with AC^{JUR} or AC^{PLB} and observed that both treatments showed upregulated NKp46 expressions, and they were significantly higher than untreated NK cells (* $P < 0.05$; ANOVA was used to compare the different.). (B) Moreover, PS-treated NK cells did not show increase cytotoxicity against autologous PHA-activated $CD4^+$ T cells. (A-B) Data are displayed as the mean ± SEM for 4 independent experiments.

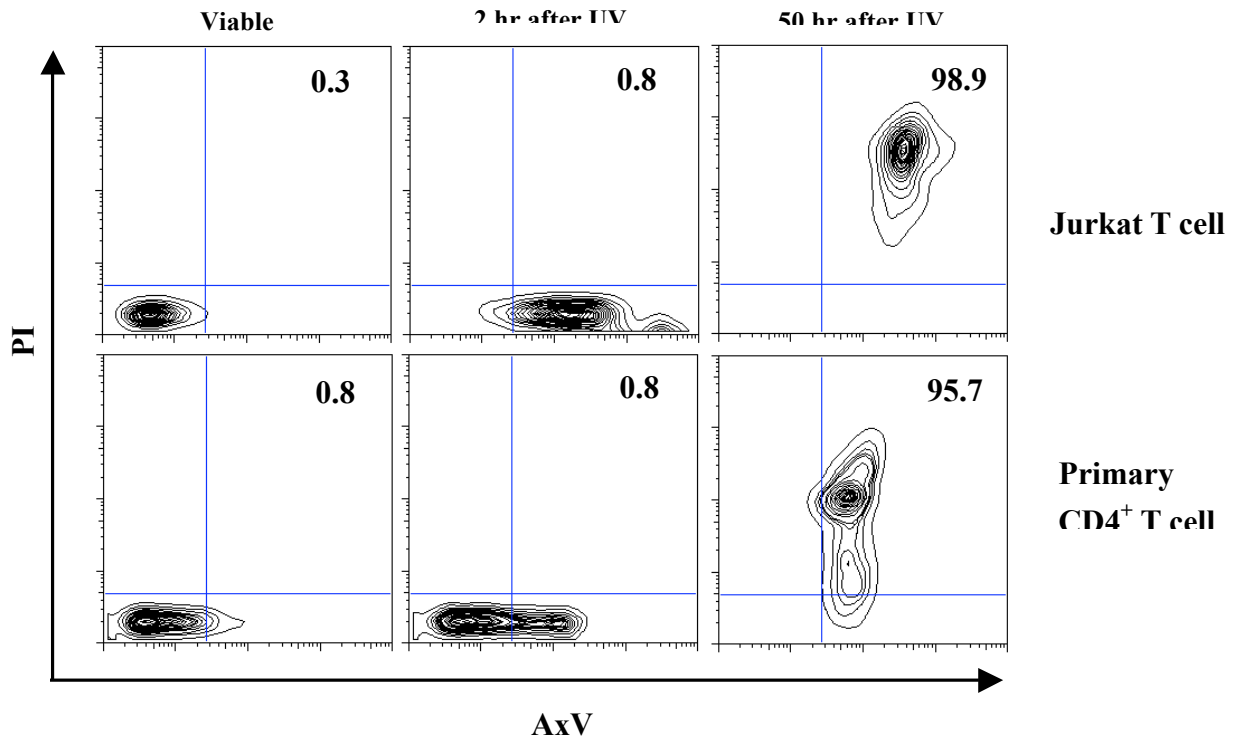**Figure S1. Induction of apoptotic cells by UV irradiation**

Figure S2. IgG opsonized AC^{JUR} but not live Jurkat T cell.

AC^{JUR}   Live Jurkat T cell

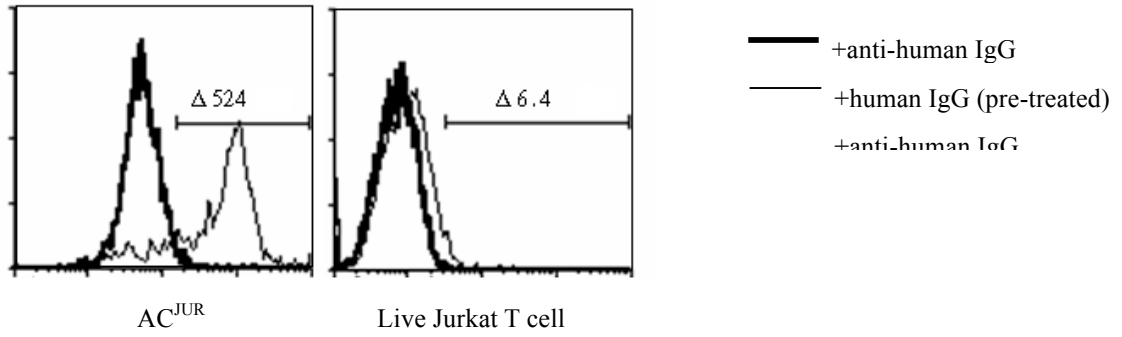— +anti-human IgG
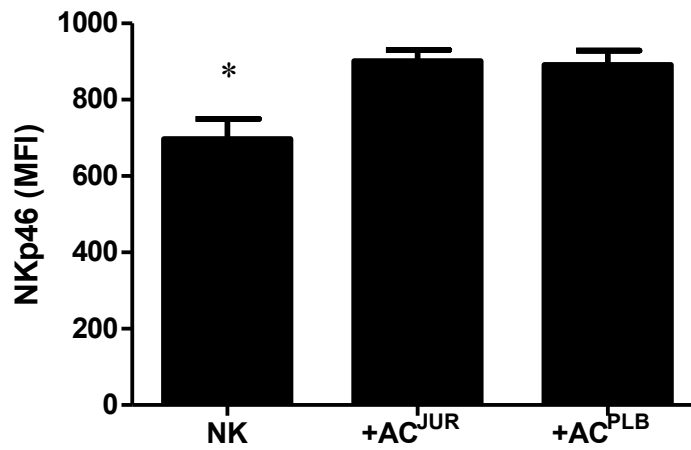
— +human IgG (pre-treated)
+anti-human IgG

Figure S3. Upregulation of NKp46 and cytotoxicity of AC-treated NK cells were independent to PS.

A

B

E (NK) : T (PHA-activated CD4$^+$ T) ratio