Molecular mechanism of the activation-induced cell death (AICD) inhibition mediated by a p70 inhibitory killer cell immunoglobulin-like receptor (KIR) in Jurkat T cells

Thesis by

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Directed by Professor Jongsun Kim

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A Dissertation for the Degree of Dr. of Philosophy in Medical Sciences by Yong-Joon Chwae has been approved by

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I' d like to thank my parents because they gave me the life and have given me the infinite graces for 34 years of my life.

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Written by Yong-joon Chwae

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Killer cell immunoglobulin-like receptors (KIRs), expressed on NK cells and a subset of $CD8^+$ T cells, transmit an inhibitory signal upon engagement with specific class I MHC molecules on target cells. $CD8^+$ T cells expressing KIRs are characterized by memory or effector phenotypes and by monoclonal or oligoclonal expansion patterns. Recent studies with KIR transgenic mice have demonstrated that KIR expression increases the survival of $CD8^+$ T cells by protecting the cells from activation-induced cell death (AICD). In this study, it has been investigated the molecular mechanisms of the AICD inhibition mediated by a p70 KIR in Jurkat T cell. Using stable Jurkat T cell lines expressing a p70 KIR (KIR3DL1, also called NKB1) and CD8-KIR fusion proteins, it was demonstrated for the first time that the p70 KIR inhibited the AICD induced by PHA or PMA/ionomycin in a ligation-independent manner. The AICD inhibition mediated by the 70 KIR appeared to be due to the blockade of FasL induction upon activating the Jurkat transfectants. According to the information that the membrane proximal 20 amino acids of the KIR cytoplasmic tail containing a putative PKC substrate site, play a crucial role in the AICD inhibition of the Jurkat T cells and the p70 KIR constitutively binds to PKCα, a conventional Ca^{2+}-dependent PKC, and PKCθ, a novel Ca^{2+}-independent PKC, it was investigated whether the p70 KIR affected PKC activation after PHA stimulation. *In vitro* kinase assay showed that PKC activation was blocked after PHA stimulation in Jurkat transfectants expressing the p70 KIR. These observations were confirmed by showing that a recombinant KIR cytoplasmic tail also inhibited the PKCα activation *in vitro*. Taken together, these data strongly suggest that KIR inhibits the AICD of T cells by blocking FasL induction upon stimulation, and this process seems to be accomplished by PKC recruitment to the membrane proximal PKC binding site and subsequent inhibition of PKC activation against activating stimuli.

Key words: KIR, AICD, Jurkat T cell, PKC, FasL

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I. Introduction

The physiological function of NK cells appears to be regulated by a delicate balance between signals transmitted through activating and inhibitory receptors on the NK cell surface, some of which interact with oligomorphic determinants of autologous MHC class I molecules.[1] Inhibitory NK cell receptors (inhibitory NKRs) consist of two broad classes of membrane anchored glycoproteins: the lectin-like receptors including Ly49 receptors of mouse[2] and CD94/NKG2 of humans, and Ig-like receptors including inhibitory killer cell Ig-like receptors (inhibitory KIRs)[3] and Ig-like transcript molecules [ILTs, also called leukocyte Ig-like receptors (LIRs)].[4,5]

Although first characterized on NK cells, inhibitory KIRs are also found on subpopulations of αβ T cells[6-8] and γδ T cells.[9,10] Both $CD4^+$ and $CD8^+$ αβ T cells express inhibitory KIRs, but KIR^+CD8^+ αβ T cells are much more commonly observed.[11] The inhibitory KIRs expressed on T cells have been shown to transmit an inhibitory signal that blocks activating signals generated from the TCR, and in turn inhibits target cell cytolysis and cytokine release.[7,8,10-17] A common feature of inhibitory KIR^+ T cells is a cell surface phenotype characteristics of memory T cells: they lack CD28 and CD45RA, mostly express CD45RO, and express high levels of CD18, CD44, CD29, and CD57.[12,18] These cells are also $CCR7^-$, like T cells belonging to the effector memory T cell subset.[19] Recent studies have suggested that inhibitory

KIR plays a role in the survival of memory-phenotype T cells[20,21] and in the inhibition of AICD of T cells.[22] However, the exact mechanism of the AICD inhibition mediated by KIR has not been revealed yet.

Inhibitory KIRs contain immunereceptor tyrosine-based inhibition motifs (ITIMs) within their cytoplasmic domain. The protein tyrosine phosphatase SHP-1/2 binds to tyrosine-phosphorylated ITIMs and subsequently dephosphorylates multiple signaling molecules involved in early stage of the activating signal transduction pathway.[23] As another signaling mechanism through ITIM motifs, it has been suggested that the phosphorylated form of p58 KIRs binds to the p85α subunit of PI3-kinase that may lead to the activation of anti-apoptotic AKT kinase.[24]

The activation of T cells via the TCR/CD3 complex leads to the increased hydrolysis of inositol phospholipids and to the subsequent production of inositol polyphosphates and diacylglycerol (DAG), which result in the elevation of intracellular calcium concentration and activation of protein kinases C (PKC), respectively.[25,26] PKC represents a family of serine/threonine-specific protein kinases. At present, 11 different PKC isoenzymes are known. On the basis of their structural and biochemical properties, they can be divided into three groups[27-29]: conventional PKC (cPKCs) α, β1, β2, and γ; novel PKC (nPKCs) δ, ε, η, and θ; and atypical PKC (aPKCs) λ, ι, and ζ. The activity of all PKC family members depends on phosphatidylserine, but some of them require additional activators such as DAG, Ca^{2+}, and PIP_3.[30] The cPKCs are activated in a DAG- and calcium-dependent manner; nPKC activity is DAG-dependent but calcium-independent [30]; aPKCs do not respond to DAG or calcium, but are activated by other lipids, such as phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P3).[31,32]

Triggering of antigen-specific TCR induces the activation of T cells, cytokine secretion, and proliferation. Once the antigens have been cleared, however, the activated T lymphocytes are removed by apoptosis to maintain T cell homeostasis.[33] This form of apoptosis is known as AICD, and it involves the TCR-induced expression of Fas ligand (FasL) on the surface of T cells. Once FasL is expressed on the T cell surface, it induces T cell apoptosis through its receptor Fas.[34-38] In addition to TCR ligation, the

combination of phorbol ester plus Ca^{2+} ionophore, which mimics the two physiological signals required for T cell activation and IL-2 production, can also induce FasL expression[39,40] and AICD.[41] Phorbol ester mediates their pleiotropic effects mainly via the activation of PKC. Thus it is highly likely that phorbol ester-sensitive PKC isoforms perform essential roles in FasL induction during AICD.[42-44]

In present work, we have investigated the molecular mechanism of the AICD inhibition mediated by inhibitory KIR in Jurkat T cells. Using stable Jurkat T cells expressing a p70 KIR (KIR3DL1, also called NKB1), it was revealed that the inhibitory KIR molecules expressed on the Jurkat transfectants inhibited the AICD independent of receptor ligation, and that the signaling mechanism of this observation was associated with the blockade of PKC activation through the membrane proximal 20 amino acids of KIR cytoplasmic tail.

II. Materials and Methods

1. Cells, antibodies and other reagents

The human leukemic T cell line, Jurkat was obtained from ATCC and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Anti-CD8 monoclonal antibody (OKT8) and anti-CD3 monoclonal antibody (OKT3) were purified from hybridoma cells using a protein A sepharose column. Anti-p70 KIR antibody (DX9) was purchased from Pharmingen (San Diago, CA, USA). Mouse anti-CD8 mAb (H-169) and rabbit anti-CD8 polyclonal Abs were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Southern Biotechnology (Birmingham, AB, USA), respectively. Apoptosis-inducing (CH11) and blocking (ZB4) anti-Fas mAbs were from Upstate Biotechnology (Lake Placid, NY, USA) and MBL (Nagoya, Japan), respectively. Anti-FasL mAb was from Pharmingen. Goat anti-mouse IgG (GAM), phytohemagglutinin (PHA), and phorbol-12-myristate-13-acetate (PMA) were from Sigma (St. Louis, MO, USA). Ionomycin was from Calbiochem (La Jolla, CA, USA).

2. Expression constructs and stable transfections

The whole p70 KIR protein coding region was amplified from pMET7-NKB1 construct[45] by PCR method and inserted into the *EcoRI*/*BamHI* sites of the pcDNA 3.1 (Invitrogen, Carlsbad, CA, USA). The fusion construct between CD8 extracellular and transmembrane domains, and p70 KIR cytoplasmic tail (pCD8KIR) were made by insertion of the PCR-amplified complete p70 KIR cytoplasmic tail sequences to the *BglII*/*BamHI* sites of the pCD8T plasmid (Fig. 1).[46] The pCD8KIR construct encodes the whole protein coding region of p70 KIR cytoplasmic tail (a.a. 361- a.a. 444). The sequences of all the constructs were verified by automatic DNA sequencing. Cells (10^7) were transfected at 500 μF/300 V using BRL (Gaithersberg, MD, USA) electroporator with 10 μg of each plasmid and selected in RPMI 1640 media containing 1 mg/ml geneticin (Sigma) for 2 weeks. Geneticin-resistent transfectants were tested for the

expression of either the p70 KIR or the CD8KIR fusion construct by FACS and Western blot analysis.

3. T cell stimulation and cell death analysis

For the induction of AICD, Jurkat T cell transfectants (5 X 10^5/ml) were stimulated either with indicated concentrations of PHA for 12 h or with 100 ng/ml PMA and 1 μg/ml ionomycin for 24 h. In the Fas-mediated apoptosis studies, Jurkat transfectants (5 X 10^5/ml) were treated with 50 ng/ml CH11, anti-Fas Ab for the indicated time periods. Apoptotic cell fractions were determined by Annexin V-FITC (Biosource International, Camarillo, CA, USA) staining described as manufacturer's instructions and analyzed on a FACScan (Becton Dickinson, Lincoln Park, NJ, USA).

4. Immunoprecipitation

Stimulated or unstimulated Jurkat transfectants (5 x 10^7 cells) were lysed using 1 ml of lysis buffer [10mM Tris-HCl, pH7.4, 150 mM NaCl, 2 mM EDTA 1 % Triton X100, 1 mM phenylmethylsufonyl fluoride (PMSF), 15 μg/ml Leupeptin, 2 mM NaF, and 2 mM $NaVO_4$] for 1 h at 4°C, then centrifuged for 15 min at 3,000 rpm and subsequently for 30 min at 13000 rpm at 4°C. The supernatants were kept at -70°C when not immediately used. The cell lysates were precleared with protein A/G-Sepharose (Pharmacia, Uppsala, Sweden) by incubation for 1 h at 4°C with constant agitation. The precleared lysates were then incubated for 1 h with appropriate Ab and protein A/G-Sepharose at 4°C. The immunoprecipitates were washed six times in the lysis buffer as above. An aliquot of each sample was taken to be analyzed by Western blots.

5. FasL mRNA expression analysis by RT-PCR

Jurkat stable transfectants (5 X 10^6 cells) were stimulated with PHA (3 μg/ml) for 4 h and total cellular RNAs were extracted using RNeasy mini kit (Qiagen, Santa Clarita, CA) and subjected to RT-PCR

analysis of FasL and HGPRT as previously described.[47]

6. Flow cytometric detection of cell surface molecules

Staining for p70 KIR, CD8, and Fas was performed by incubating Jurkat transfectants with saturating amounts of DX9, OKT8, and anti-Fas Ab, respectively, in phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) for 30 min at 4°C. Expression level was measured on a FACScan (Becton Dickinson) and analyzed with the WinMDI program (Joseph Trotter).

7. Flow cytometric detection of cytoplasmic FasL

Intracellular content of FasL was measured in Jurkat T cell transfectants before and after stimulation.in the presence of GolgiStop, an inhibitor of protein secretion that results in the cytoplasmic accumulation of the synthesized FasL. After cell fixation and permeabilization, intracellular staining was performed according to the method described by Baars et al.[48] Briefly, Jurkat T cells ($5X10^5$ cells/ml) were stimulated for 4 h with PHA (3 μg/ml) in the presence of GolgiStop. Then, the cells were washed twice in cold PBS containing 0.1% BSA and fixed with PBS containing 4% paraformaldehyde for 5 min at 4°C. Fixation was followed by permeabilization with PBS containing 0.1% saponin (Calbiochem) and 0.5% BSA. Nonspecific binding was blocked by incubating the cells in the same buffer supplemented with 10% human serum for 20 min at 4°C. For all subsequent incubation and washing steps, PBS containing 0.1% saponin, 0.5% BSA was used. Cells were then washed once and stained with 5 μg/ml anti-FasL Ab for 30 min at 4°C. After another washing step, cells were stained with FITC-labeled goat anti-mouse IgG mAb for 20 min at 4°C. Expression levels of FasL were measured on a FACScan described as above.

8. PKC kinase assay

The PKC kinase assay was performed using SignaTECT protein kinase C assay sytem (Promega,

Madison, WI) described as manufacturer's instructions with minor modifications. Briefly, PKC immunoprecipitates obtained from 5 X 10^6 Jurkat cell transfectants were incubated with 100 μM of biotinylated PKC-selective synthetic peptide (NH_2-AAKIQASFRGHMARKK- COOH) in a kinase reaction buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.25 mM EGTA, 0.4 mM $CaCl_2$, 1 mg/ml BSA, 0.1 mM ATP, 0.5 μCi [γ-^{32}P]ATP) for either 10 min (PKCα) or 30 min (PKCθ) at 25°C in a final volume of 25 μl. The reaction was stopped by centrifugation at 3000 X g for 5 min, which separated the immobilized PKC immunoprecipitates from the soluble substrate. 10 μl of supernatant from each sample was spotted onto SAM^{2R}Biotin capture membrane, washed 4 times with 2 M NaCl and 4 times with 2 M NaCl containing 1% phosphoric acid, and the incorporation of ^{32}P into peptide was detected by liquid scintillation counting.

9. Preparation of bacterial recombinant p70 cytoplasmic tail

The His-tag fusion protein of a p70 KIR cytoplasmic tail was prepared as described previously.[49,50] Briefly, the protein coding region of the cytoplasmic tail of p70 KIR (KIR-cyt) was subcloned into an *E coli* expression vector, pRSETA (Invitrogen), and the plasmids were transformed into E. coli BL21 (DE3) plyS for expression. Six liters of bacteria carrying expression plasmids were grown from a single colony. 4 h after induction with isopropyl-β-D-thiogalactopyranoside (IPTG), bacteria were harvested by centrifugation, and the inclusion bodies (insoluble protein aggregates) were isolated as described by Nagai and Thogerson,[51] with the modification of a freeze-and-thaw step after detergent treatment of bacteria. Refolding of the denatured KIR-cyt protein was performed by a dilution method in the presence of a reduced/oxidized glutathione redox buffer. The refolding mixture was concentrated and subjected to a FPLC gel-filtration column (Pharmacia) for purification.

II. Results

1. KIR expression inhibits the AICD of Jurkat T cells in a ligation-independent manner.

To investigate the effect of KIR on the AICD of T cells, a p70 KIR (KIR3DL1, also called NKB1) full-length cDNA was stably transfected to the Jurkat T cells. Jurkat T cell line has been widely used as a good model of T cell AICD studies since the AICD can be easily induced by various activating signals. Expression levels of the p70 KIR in Jurkat transfectants were measured by FACS analysis (Fig. 2A), and three independent clones expressing the different levels of the p70 KIR were selected and used for AICD study of Jurkat T cells. Interestingly, it was found that the p70 KIR expressing cells appeared to be larger than control cells. For unknown reason, the cell surface area increased more than two times (data not shown). Growth rates of the p70 KIR transfectants were also compared with the control transfectant and it was found that Jurkat cells expressing full-length p70 KIR grew similarly at the log phase, but reached at the stationary phase much faster with less cell density than the control cells (Fig. 2B). These observations suggest that KIR might constitutively transmit a signal that affects cell size and growth pattern in KIR transfectants.

The process of AICD can be mimicked in Jurkat T cells by cross-linking the TCR complex with anti-CD3 antibodies or phytohemagglutinin (PHA), or by activating downstream signaling molecules, such as protein kinase C and calcineurin, with phorbol esters (PMA) in combination with a calcium ionophore (ionomycin).[52-54] To induce AICD, the Jurkat transfectants expressing the p70 KIR were stimulated with PHA. Surprisingly, Jurkat transfectants expressing the p70 KIR showed much less apoptotic cell death than Jurkat control and vector control. The differences of cell death patterns between control Jurkat and KIR-expressing Jurkat were more obvious at lower concentrations of PHA than at higher concentration (Fig. 3A). Inhibitory effect of the p70 KIR expression on Jurkat AICD was dependent on to the expression levels of KIR molecule on the surface of Jurkat: Jurkat transfectants

expressing KIR molecules at higher level showed less apoptotic cell deaths than Jurkat transfectants expressing KIR molecules at lower level (Fig. 3B).

Previous studies have suggested that cross-linking of KIR inhibited the target cell-lysis and IL-2 secretion of cytotoxic T lymphocytes (CTLs) when the CTLs were stimulated.[7,8,10-17] To investigate the effect of KIR cross-linking on the AICD of Jurkat T cells expressing the p70 KIR, Jurkat transfectants were pretreated with an anti-p70 KIR monoclonal antibody (DX9) and cross-linking anti-mouse IgG antibody (GAM) before PHA stimulation. Interestingly, however, cross-linking of p70 KIR by DX9 did not affect the AICD inhibition mediated by KIR (Fig. 4A). This result suggests that KIR might function in a ligation-independent manner to inhibit the T cell AICD. To confirm this observation, a CD8KIR fusion construct that encodes the extracellular and transmembrane domains of CD8 α chain and the cytoplasmic tail of p70 KIR was made. Consistent with the above result, Jurkat transfectants expressing the CD8KIR fusion protein also appeared to be less sensitive to the AICD regardless of the receptor cross-linking with OKT8 antibody (Fig. 4B). These results indicate that KIR expression inhibits the AICD in a ligation independent manner in Jurkat T cells.

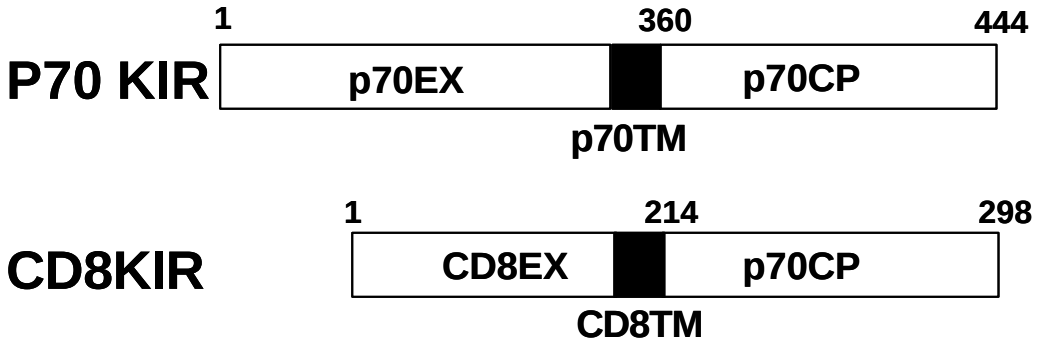


Figure 1. Schematic diagram of p70KIR and CD8KIR. These constructs were subcloned into the pcDNA3.1 (Invitrogen) and transfected into Jurkat cells. Black boxes represent the transmembrane region. p70KIR construct consists of extracellular domain (p70EX), transmembrane domain (p70TM), and cytoplasmic tail (p70CP) of the p70 KIR. CD8KIR construct consists of extracellular domain (CD8EX) and transmembrane domain (CD8TM) of CD8α chain, and cytoplasmic tail of the p70 KIR

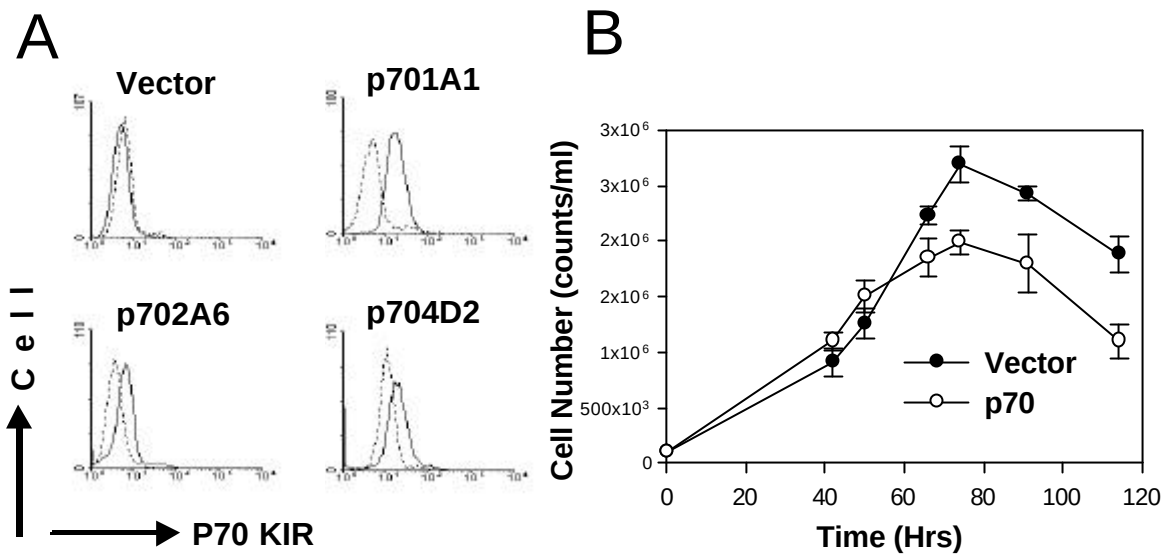


Figure 2. Establishment of Jurkat stable cell lines expressing the p70 KIR. (A) Expression of the p70 KIR on the surface of Jurkat transfectants. The protein coding region of p70 KIR was cloned into pcDNA 3.1, and the recombinant DNA was stably transfected into Jurkat T cells. Among the clones resistant to Geneticin, three independent clones expressing the p70 KIR on the surface were selected (p701A1, p702A6, p704D2). Expression levels were measured using FACS analysis with DX9 mAb. (B) Growth curves of Jurkat transfectants expressing the p70 KIR and vector control. Jurkat clones (Jurkat transfectants expressing the p70 KIR and vector control) were seeded in 96 well plates at the concentration of 5 X 10^4 cells/ml and cell numbers were counted at the indicated time with the trypan blue dye exclusion method.

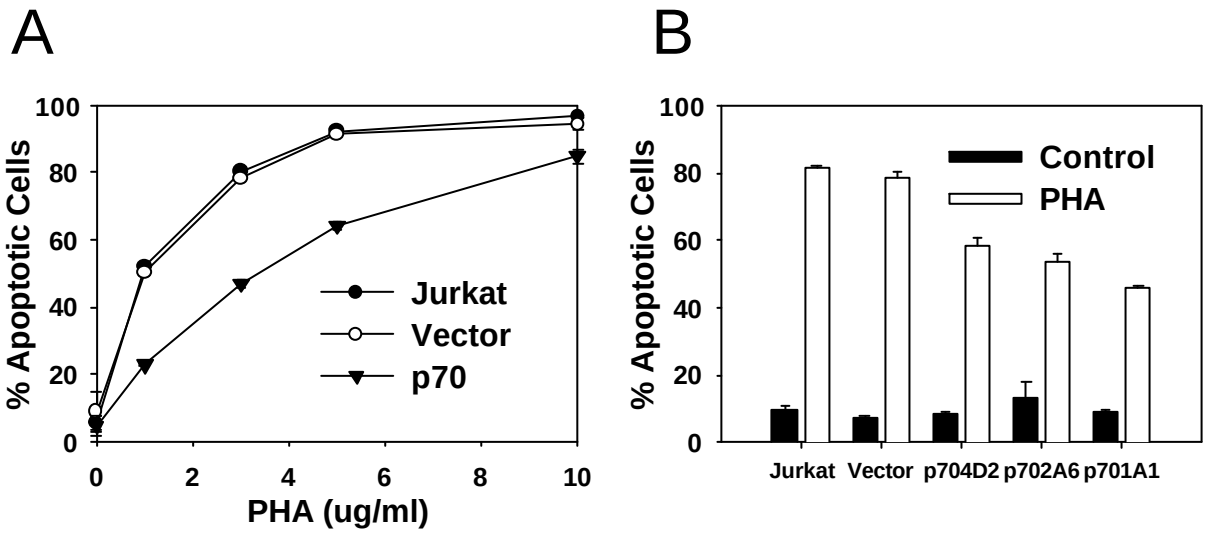


Figure 3. p70 KIR expression inhibits the AICD of Jurkat T cells. (A) AICD of Jurkat, vector control, and Jurkat expressing the p70 KIR induced by the various concentrations of PHA. Jurkat cells (5 X 10^5

cells) were placed in the 24 well plates and PHA was added to the concentrations of 0, 1, 3, 5, 10 μg/ml. After 12 h incubation, apoptotic cell death ratios were determined using Annexin V staining as described in the "Materials and Methods". (B) Inhibitory effect of the KIR expression on Jurkat AICD is proportional to the expression level of the KIR. Three independent clones expressing different levels of the p70 KIR were tested for AICD by treating 3 μg/ml PHA. Results were mean ± SD values of three independent experiments, done with duplicate samples.

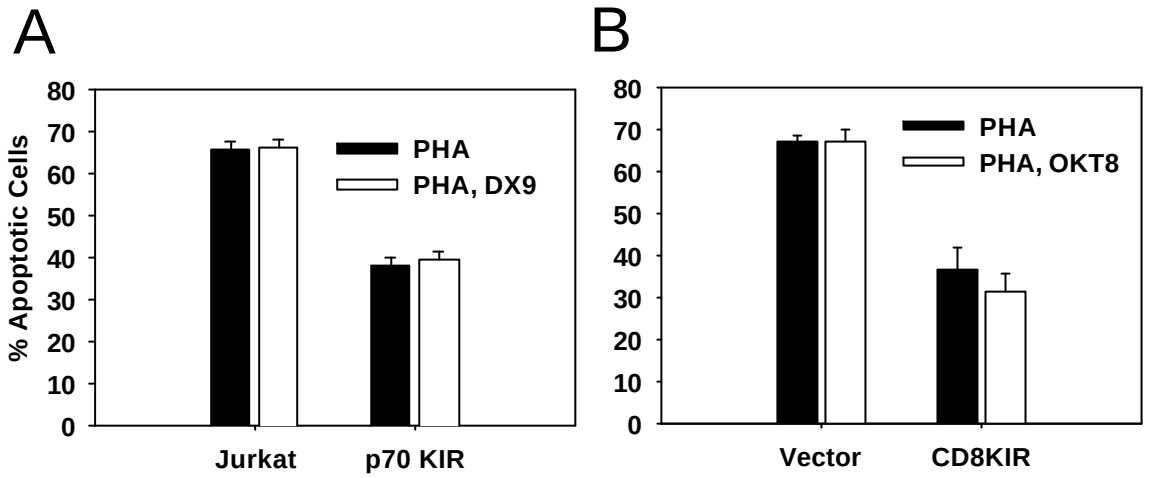


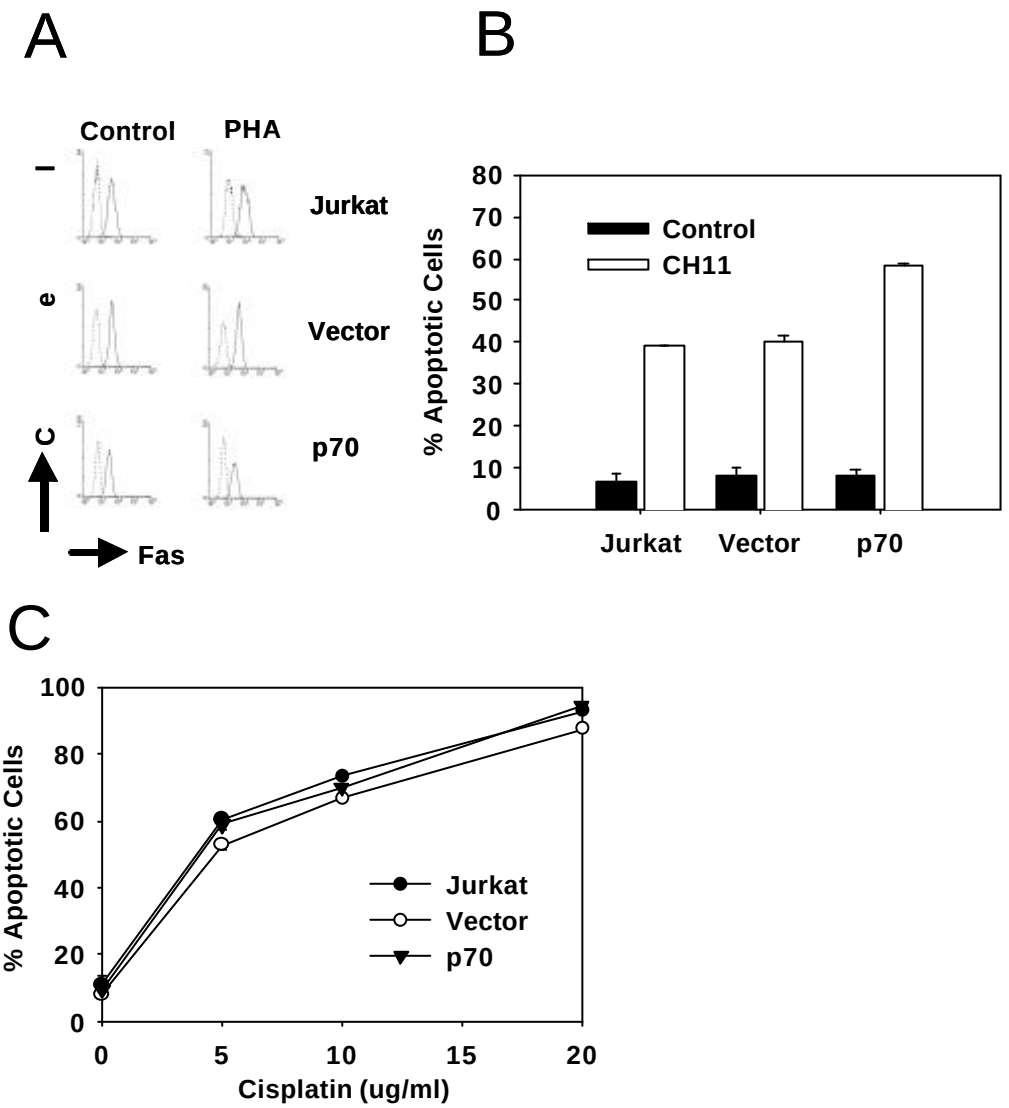
Figure 4. AICD inhibition in the Jurkat transfectants expressing the KIR is independent of receptor ligation. (A) Effects of the KIR cross-linking on the PHA-induced AICD of Jurkat and Jurkat transfectant expressing the KIR. Jurkat control and Jurkat transfectant expressing the KIR were incubated with or without 5 μg/ml DX9 an anti-p70 KIR mAb, and cross-linked with 10 μg/ml goat anti-mouse IgG Ab. After 12 h incubation, cell deaths were measured as described in "Materials and Methods". (B) Effects of CD8 cross-linking on the PHA-induced AICD of Jurkat transfectants expressing the CD8KIR fusion protein. Jurkat T cells transfected with an empty vector or a CD8KIR construct were incubated with or without 5 μg/ml OKT8 mAb and cross-linked with 10 μg/ml goat anti-mouse IgG Ab. Cell death was induced by 3 μg/ml PHA treatment and measured after 12 h incubation. Each experiment was performed with three independent clones and the result shown above was that of one representative clone, each done with duplicates.

2. KIR expression protects Jurkat T cells from AICD via inhibiting FasL expression.

It has been previously demonstrated that AICD proceeds via induction of FasL expression and subsequent Fas/FasL interaction on the surface of activated T cells.[35,55] Therefore, the observed inhibition of AICD by KIR expression could conceivably be mediated by at least 4 nonexclusive mechanisms: by inhibition of FasL expression, by inhibition of Fas receptor expression, by inhibition of apoptotic signaling events downstream of Fas ligation, or by inhibition of common apoptotic signaling pathway. The expression level of Fas on the surface of p70 KIR transfectants by flowcytometry analysis before and after the PHA stimulation was examined. As shown in Figure 5A, Fas expression was not significantly changed in Jurkat transfectants expressing KIR compared with control Jurkat cells and the expression level was not changed even after the PHA stimulation. To evaluate whether the downstream apoptotic signaling events after Fas ligation is defective in KIR transfectants, Fas-mediated apoptosis of the Jurkat transfectants was induced by CH11, agonistic anti-Fas mAb. As shown in Figure 5B, CH11 effectively induced apoptotic cell death in Jurkat transfectants expressing KIR, as well as in control Jurkat cells. Interestingly, Jurkat transfectants expressing KIR appeared to be more sensitive to the Fas-mediated apoptosis. Next, it was evaluated whether Jurkat transfectants expressing KIR have some defects in common apoptotic signaling pathway. For this purpose, a cytotoxic drug, cisplatin was used to induce apoptotic cell death and it was found that KIR did not affect the apoptosis mediated by the cytotoxic drug (Fig. 5C).

Next, the expression level of FasL by RT-PCR and cytoplasmic FACS analysis in the presence of GolgiStop, a monesin-based protein secretion inhibitor was examined. Cytoplasmic FasL expression was not detected in both Jurkat control and Jurkat expressing the p70 KIR before PHA stimulation (data not shown). After PHA (3 μg/ml) treatment, FasL expression was strongly induced in Jurkat T cells, whereas FasL induction in Jurkat transfectants expressing the p70 KIR was almost completely blocked at both mRNA and protein levels (Fig. 6A and 6B). Then, FasL blocking experiment with anti-Fas antibody was

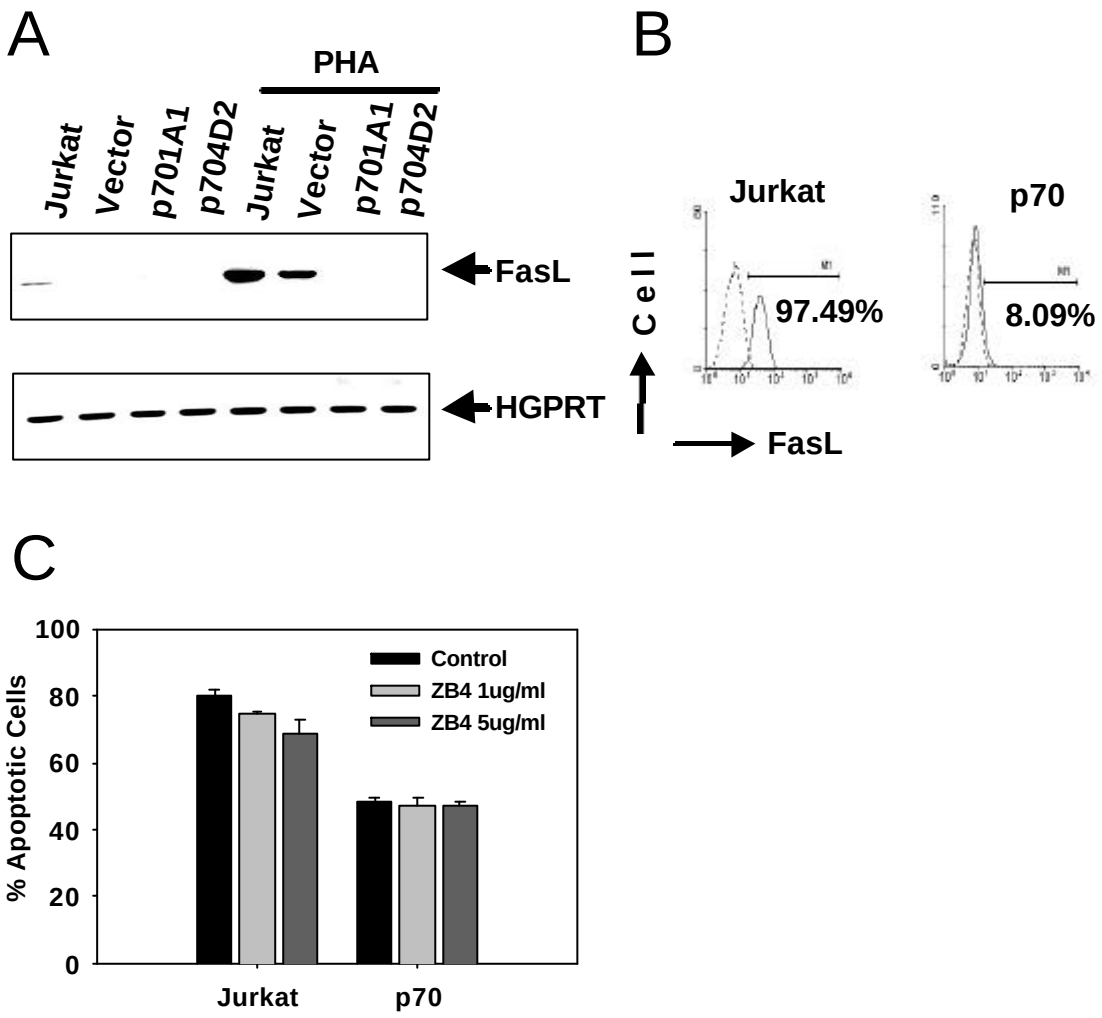
performed. As expected, pretreatment of anti-Fas blocking Ab, ZB4 significantly blocked the cell death of the Jurkat cells upon activation with PHA in a dose-dependent manner. In contrast, in the Jurkat transfectants expressing KIR this effect was not observed (Fig. 6C). Taken together, these results clearly demonstrate that the inhibitory effect of Jurkat AICD via KIR expression is mediated by inhibition of



FasL induction.

Figure 5. AICD inhibition in the Jurkat transfectants expressing the KIR is not associated with the change of Fas expression level, nor with the defect in downstream signal of Fas. (A) Fas expression of Jurkat and Jurkat transfectants before and after PHA stimulation. Cells were stimulated with 3 μg/ml PHA and for 3 h, and the changes of Fas expression level were checked by flow cytometry. (B) Fas-mediated apoptosis

of Jurkat transfectants. Jurkat transfectants were treated with 50 ng/ml CH11, anti-Fas mAb for 12 h. Fas-mediated apoptosis was measured by Annexin V staining as describe in the "Materials and Methods". (C) Apoptosis of Jurkat transfectants induced by cisplatin. Jurkat transfectants (5 X 10^5 cells/ml) were treated with cisplatin of various concentrations (0, 5, 10, 20 μg/ml for 24 h, and tested for apoptotic cell death



using Annexin V staining as describe in the "Materials and Methods". The data represent the mean ± SD of three independent experiments, each done with duplicate.

Figure 6. AICD inhibition in the Jurkat transfectants expressing the KIR is associated with inhibition of FasL induction. (A) FasL mRNA expression of Jurkat transfectants expressing the KIR, Jurkat, and vector control before and after PHA stimulation. Jurkat cells (4 X 10^6 cells) were treated with 3 μg/ml PHA for 3 h and FasL RT -PCR was performed as described in the "Materials and Methods". Upper panel shows the PCR-amplified FasL mRNA and lower panel shows the PCR amplified control HGPRT mRNA. (B) Cytoplasmic FasL expression of Jurkat transfectant expressing the KIR and Jurkat after PHA stimulation. Jurkat cells (1 X 10^6) were pre-treated with GolgiStop, a monesin-based inhibitor of protein secretion, before 3 μg/ml PHA treatment for 3 h, and intracytoplasmic FasL staining was performed with

anti-FasL Ab as described in the "Materials and Methods". (C) Blocking of AICD by anti-Fas blocking Ab. Anti-Fas blocking Ab, ZB4 was pre-treated for 1 hr at the indicated concentrations before PHA treatment (3 μg/ml), and 12 h later, apoptotic cell deaths were measured by Annexin V staining as described in the "Materials and Methods". The data represent the mean ± SD of three independent experiments performed duplicates each time.

3. AICD induced by PMA and ionomycin treatment is also blocked in Jurkat transfectants expressing KIR.

The present data showed that the AICD induced by PHA stimulation is significantly inhibited in Jurkat transfectants expressing the p70 KIR (Fig. 3) and the inhibition of AICD is mediated by blocking the FasL induction (Fig. 6). Similar phenomena were also observed when AICD was induced by a direct stimulation of TCR using an anti-CD3 mAb (data not shown). As the first step to understand the molecular mechanism of these observations, it was investigated whether the Jurkat transfectants expressing the p70 KIR would also be resistant against AICD induced by PMA and ionomycin. PMA and ionomycin, which are known as a PKC activator and calcium ionophore, respectively, have been widely used to bypass the early TCR signaling pathway by direct stimulation of PKC and calcium signaling pathway in T cells. As shown in the Figure 7, when Jurkat transfectants were stimulated with 100 ng/ml, PMA and 1 μg/ml, ionomycin, Jurkat transfectants expressing the p70 KIR also exhibited significantly less apoptotic cell death compared to Jurkat control and vector control (12.90 ± 0.28 % versus 31.30 ± 0.99 % and 36.50 ± 1.41 %, respectively). This suggests that the inhibition of Jurkat T cell AICD by the p70 KIR expression may result from the inhibition of PKC and/or calcium signaling pathway(s).

Bearing in mind of the above observation, it was reexamined the amino acid sequences of the KIR cytoplasmic tails. Interestingly, it is found that all inhibitory KIR family members have three putative PKC phosphorylation sites in the cytoplasmic tails. The first one is located at the membrane-proximal region and the other two are between the two ITIMs (Fig. 8).

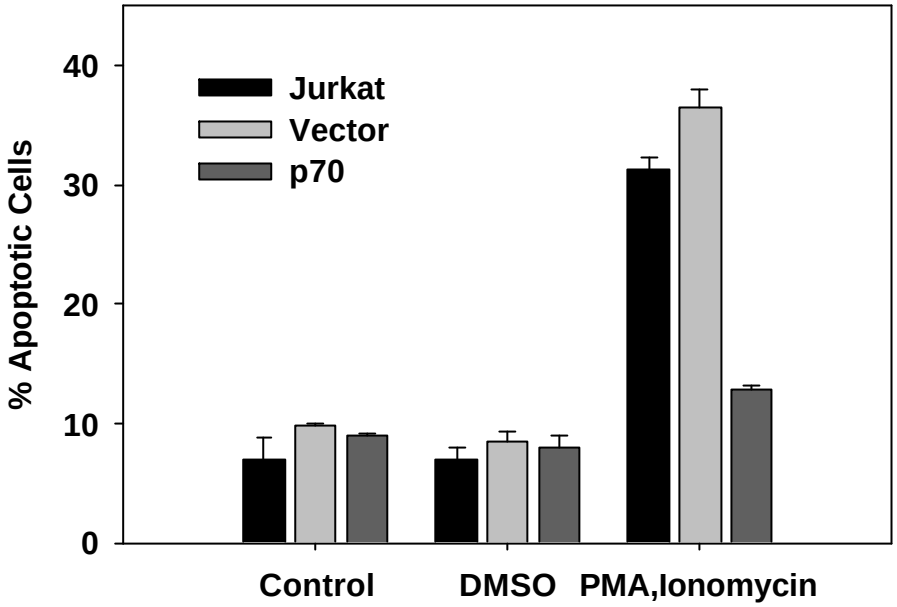


Figure 7. Jurkat transfectants expressing the KIR are also resistant to PMA and ionomycin-induced AICD. Jurkat, vector control, and Jurkat transfectants expressing the KIR were treated with or without 100 ng/ml PMA and 1 μg/ml ionomycin, or equivalent volume of DMSO (0.1%) as the solvent control. Apoptotic cell death was detected with Annexin V staining as described in the "Materials and Methods". The data represent mean ± SD of three independent experiments.

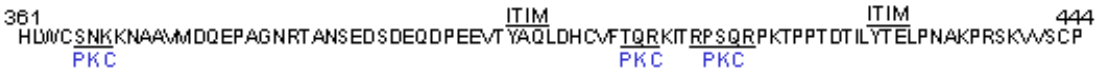


Figure 8. Amino acid sequences of the p70 KIR cytoplamic tail. Amino acid sequences of the p70 KIR cytoplamic tail is shown into the single letter code. Putative PKC binding sites are underlined and ITIM motifs are overlined.

4. PKCa and PKCq activations induced by PHA stimulation are blocked in Jurkat transfectants expressing the cytoplasmic tail of p70 KIR.

Next, it was investigated whether PKCs could be normally activated after PHA stimulation in Jurkat transfectants expressing the p70 KIR. *In vitro* PKC kinase assay was performed using PKCα and PKCθ immunoprecipitates obtained from Jurkat and Jurkat transfectants at various time intervals after PHA

stimulation. In the control Jurkat cells, PKCα was activated as early as 5 min after PHA (3 μg/ml) treatment and reached the highest peak at 10 min, and prolonged till 60 min with continuous decrease. Interestingly, however, PKCα activation was not appeared in Jurkat transfectants expressing the p70 KIR (Fig. 9A). Similar phenomena were observed in the case of PKCθ. PKCθ was activated in control Jurkat cells after PHA treatment, but not in Jurkat transfectants expressing the p70 KIR (Fig. 9B).

5. Recombinant KIR cytoplasmic tail partially blocks the *in vitro* PKCa activation induced by phosphatidyl serine and DAG.

It was observed that KIR appeared to inhibit the activation of PKCs. To confirm this observation, the *in vitro* kinase assay using a bacterially expressed His-tag fusion protein of the p70 KIR cytoplasmic tail was performed.[49,50] PKCα proteins were prepared from resting Jurkat T cells by immunoprecipitation, and activated by adding phosphatidyl serine and DAG in the presence or absence of the recombinant KIR cytoplasmic tail protein. As shown in Figure 9C, recombinant KIR cytoplasmic tail inhibited the PKCα activation induced by phosphatidyl serine and DAG treatments in a dose-dependent manner. In the presence of 10 μM recombinant KIR cytoplasmic tail, about 20% of total PKCα activity was decreased. However, BSA treatment did not affect the PKCα activation. This implies that the cytoplasmic tail of KIR has a potential to inhibit the PKC activation.

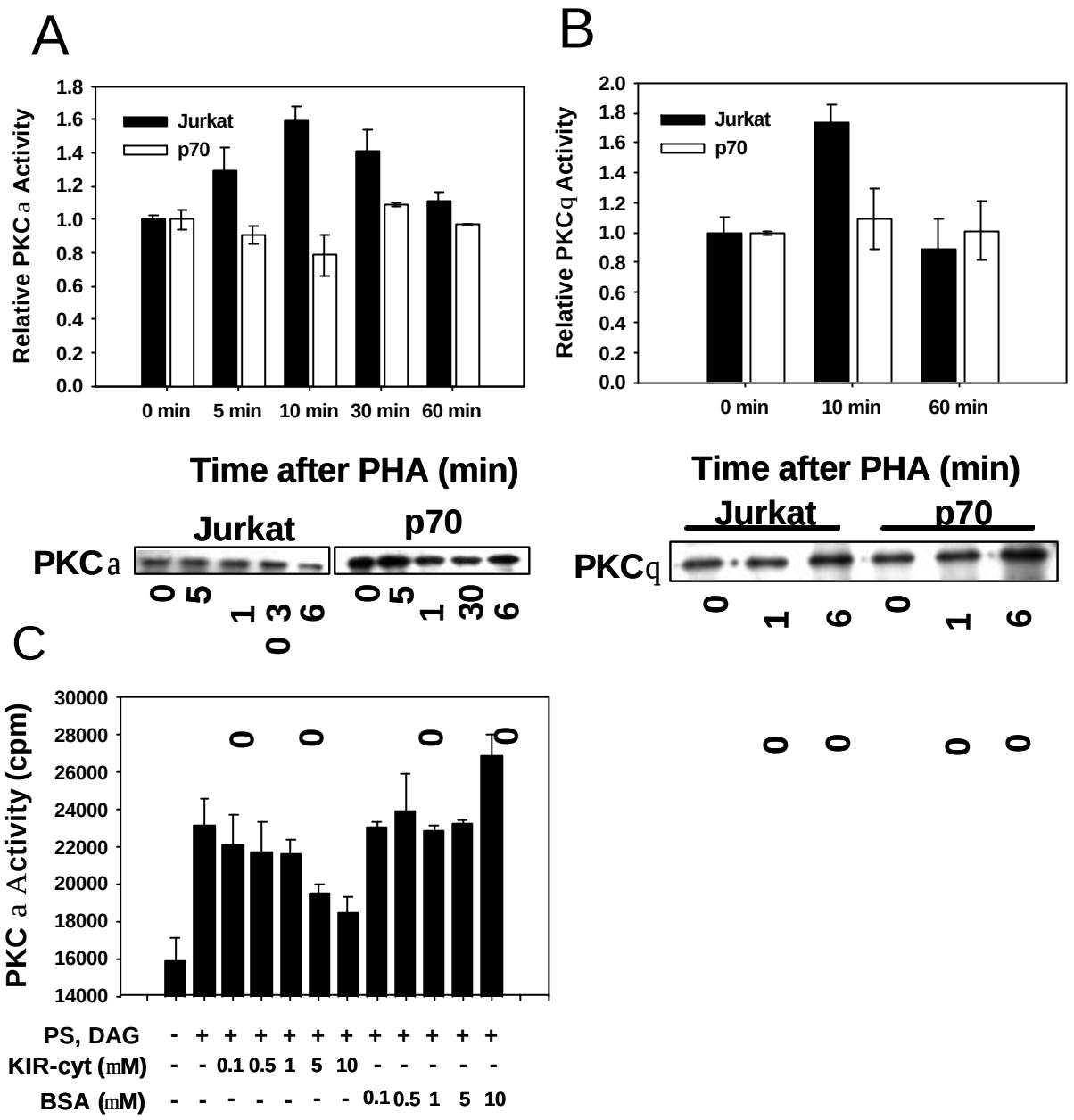


Figure 9. PKC activation is blocked by the KIR cytoplasmic tail. (A-B) Activation of PKCα and θ induced by PHA treatment in Jurkat transfectants expressing the KIR and Jurkat T cells. 1 X 10^7 cells of Jurkat and Jurkat transfectants expressing the KIR were stimulated with 3 μg/ml PHA for the indicated period of time. Cells were lysed, and PKCα and PKCθ were immunoprecipitated. Immunoprecipitates equivalent to 2 X 10^6 cell lysates were subjected to an *in vitro* kinase assay using a biotinylated PKC-selective synthetic peptide (NH_2-AAKIQASFRGHMARKK-COOH in the single-letter amino acid code) as described in the "Materials and Methods". Upper panel indicates PKC activity presented as the fold increases compared with buffer-treated control cells. The average values of three independent

experiments were shown, and the error bar represents SD of the mean. Lower panels are western blots of the PKCα and PKCθ immunoprecipitates that were used in the kinase assay. (C) Effect of the recombinant KIR cytoplasmic tail on *in vitro* PKCα activation induced by phosphatidyl serine and diacylgylcerol. PKCα immunoprecipitates were prepared from resting Jurkat T cells and activated *in vitro* by treating 0.32 mg/ml phosphatidyl serine (PS) and 0.032 mg/ml diacylgylcerol (DAG) in the presence or absence of the recombinant KIR cytoplasmic tail (KIR-cyt). BSA was used as a control protein. *In vitro* PKC assay was performed as described in the "Materials and Methods".

IV. Discussion

To better understand the immunological function of KIR in T cells, it has been investigated the effect of KIR expression on the AICD and its signaling mechanism in Jurkat T cells. In this study, it has been shown for the first time that KIR expressed on the surface of the Jurkat T cell inhibits the AICD induced by either PHA or PMA/ionomycin in a ligation-independent manner. The AICD inhibition mediated by KIR appears to be due to the blockade of FasL induction and PKC activation after the activation stimuli. To determine which region of KIR cytoplasmic tail is responsible for the KIR-mediated AICD inhibition in Jurkat T cells, a series of CD8-KIR fusion constructs was made. Surprisingly, by deletion of membrane proximal 20 amino acids including one putative PKC substrate site, the inhibitory effect of Jurkat AICD mediated by the p70 KIR cytoplasmic tail disappeared in Jurkat transfectants. These results indicate that the membrane proximal region extending 20 amino acids in p70 KIR cytoplasmic tail contains an important signaling motif responsible for the ligation independent inhibition of AICD, and the inhibitory effect may be associated with inhibition of PKC or downstream signaling pathway of PKC (our unpublished data). To elucidate the role of PKC in the KIR-mediated AICD inhibition, it was examined whether PKC bound to p70 KIR cytoplasmic tail before and after PHA stimulation using immunoprecipitation experiments. Among the PKC isoforms, PKCα and PKCθ were chosen as the representatives of conventional Ca^{2+}-dependent PKC and novel Ca^{2+}-independent PKC, respectively. As a result, PKC α and PKC θ constitutively bound to p70 KIR cytoplasmic tail, and the binding did not appear to be affected after PHA stimulation (our unpublished data). As a next step for elucidating relationships between KIR and PKC, it has been investigated whether KIR affects PKC activation after PHA stimulation. *In vitro* kinase assay results revealed that PKC activation was blocked after PHA stimulation in the Jurkat transfectants expressing KIR. These observations were confirmed by showing that a bacterially expressed recombinant KIR cytoplasmic tail also inhibited the PKCα activation *in vitro*. Taken together, these data strongly suggest that KIR inhibits the AICD of T cells by blocking

FasL induction upon stimulation, which processes seem to be accomplished by PKC recruitment to the membrane proximal PKC binding site and subsequent inhibition of PKC activation against the activating stimuli.

It is well known that cytotoxic function of killer cells is inhibited by inhibitory KIRs expressed on NK cells and some cytotoxic T cells via specific recognition of class I MHC molecules on target cells. This ligation-dependent inhibitory effect of KIR is accomplished by phosphorylation of tyrosine residue(s) in the cytoplasmic tail of KIR and by subsequent recruitment of protein tyrosine phosphatase, SHPs to the ITIM motifs.[23] Present data in this study indicate that KIR expressed on Jurkat T cells inhibits the AICD of these cells in a ligation-independent manner. Interestingly, these inhibitory function of KIR on T cell AICD is mediated by the putative PKC phosphorylation site at the membrane proximal region of KIR cytoplasmic tail, that is far from the ITIM motifs. Therefore, it is highly likely that KIRs function as a negative regulator of T cell cytotoxicity and AICD through two distinct mechanisms that are ligation-dependent and ligation-independent, respectively.

At the cytoplasmic domain of p70 KIR, there exist three putative PKC substrate sites and the membrane-proximal one appears to be critical for the recruitment of PKC and subsequent inhibition of PKC activation. The membrane-proximal PKC site shows a 100% sequence homology with all inhibitory KIRs reported so far, even with activating KIRs that lack ITIMs at the cytoplasmic tail. The functional significance of the membrane proximal region of KIR in T cell AICD might be the reminiscent of that of CTLA-4, in which the membrane proximal region is critical for the inhibitory role of this receptor in T cells.[56] Furthermore, a strictly conserved cysteine residue is located next to the serine residue of the membrane proximal PKC site (Fig. 8). Thus the primary structural features of the membrane-proximal PKC site are highly analogous to the N-myristylated PKC peptide analog frequently used for the inhibition of PKC activation *in vitro*.[57] This peptide analog is known as an irreversible PKC inhibitor in which a cysteine residue is substituted for the phosphorylable threonine residue. It has been suggested

that the covalent-linkage between the cysteine residue of the peptide analog and that of the PKC active site induce an irreversible inactivation of PKC *in vitro*.[57] It has been postulated that similar mechanism might be involved in PKC inhibition by KIR cytoplasmic tail, and present data suggest that the cytoplasmic tail of KIR may directly inhibits PKC activation through the membrane proximal PKC binding site.

AICD is defined as apoptosis of lymphocytes by any signal that results in lymphocyte activation and, in particular, by stimulation of TCRCD3 complex (or B cell receptor complex) with antigens or antibodies.[58] Several studies have shown that the expression and interaction of Fas and FasL, which results in autocrine activation of Fas-mediated apoptosis, is required for AICD of T cell hybrdomas, Jurkat T cells, and activated T cells,[33-36] and that the induction of FasL expression during AICD has been shown to be the mechanism by which other signaling molecules (CD3-ζ, Lck, ZAP-70, CD45, Ras, PKC, and calcineurin) regulate AICD.[40,43,59-63] In this context, PKC functions as important signaling molecules that regulate many transcription factors acting on the FasL promoter. In particular, PKC θ participates in NF-κB activation through activation of IκB kinase β,[64] functions as a selective upstream regulator of JNK[65] and consequently regulates AP-1 activation,[66] and affects NF-AT activation in cooperation with calcineurin.[43] PKC has also been implicated in the activation of ERK.[67] It was also observed that NF-AT promoter activity was almost completely blocked and ERK-1/2 activations were significantly decreased when the Jurkat transfectants expressing p70 KIR were stimulated with either PHA or anti-CD3 antibody (our unpublished data). These results support the present suggestion that KIR might inhibit FasL induction through the inhibition of PKC activation.

PKC is known to play an important role in Fas-mediated apoptosis, as well as in T cell activation and AICD. Interestingly, PKC appears to protect T cells from Fas-mediated apoptosis through phosphorylation of Bad[68,69] or through modulation of K^+ loss and cell shrinkage[70]. These findings provide the molecular mechanisms to explain the present observation that Jurkat transfectants expressing

p70 KIR appeared to be more susceptible than control Jurkat T cells to Fas-mediated apoptosis (Fig. 5B). Suppression of PKC activity by KIR might result in the inhibition of Bad phosphorylation and/or the modulation of K^+ loss and cell shrinkage, and consequently lead the KIR expressing Jurkat transfectants to become more susceptible to Fas-mediated apoptosis. It is possible that, although this phenomenon has a harmful effect on KIR^+ T cells, when they encounter cells expressing FasL such as some tumor cells, the effect may be ameliorated by ligation-dependent activation of PI3-kinase via ITIM motifs as has been previously suggested by Marti et al.[24]

KIR^+ $\alpha\beta$ T cells are preferentially found within antigen-experienced subsets,[18] thus suggesting selective KIR induction or expansion of KIR^+ T cells during *in vivo* T cell responses. However, direct evidence for *in vitro* or *in vivo* induction of KIR on mature lymphoid cells is still lacking. Recently, Huard et al[71] have reported that expression level of KIR on T cells is dynamically regulated through TCR-mediated up-regulation and KIR-mediated down-regulation. In line with this, inhibitory effects of KIR on T cells have been shown to be unexpectedly complex.[23,72] Therefore, it seems likely that specific time point and expression level of KIR on the surface of T cells would be important factors for the function of KIR, especially for the ligation-independent PKC inhibition via KIR.

In summary, it has been demonstrated by this work that KIR inhibits the AICD of Jurkat T cells in a ligation-independent manner via the inhibition of FasL induction and that the process seems to be mediated by the inhibition of PKC activation through the membrane proximal region of KIR cytoplasmic tail rather than ITIM motifs. Future studies should be focused on the elucidation of the exact molecular mechanisms of the PKC inhibition mediated by KIR. Thereby, this will help us to better comprehend the complex roles of KIR in T cells.

V. Conclusions

In this study, the molecular mechanisms of the AICD inhibition mediated by a p70 KIR in Jurkat T cells were investigated. Using stable Jurkat T cell lines expressing the p70 KIR and CD8-KIR fusion proteins, it was demonstrated for the first time that the p70 KIR inhibited the AICD induced by treatment of either PHA or PMA/ionomycin in a ligation-independent manner and that these effects appeared to be due to the blockade of FasL induction upon activation of the Jurkat transfectants. According to the information that the membrane proximal 20 amino acids of the KIR cytoplasmic tail containing a putative PKC substrate site, play a crucial role in the AICD inhibition of the Jurkat T cells and that the p70 KIR constitutively binds to PKCα and PKCθ, it was investigated whether the p70 KIR would affect PKC activation after PHA stimulation. *In vitro* kinase assay results revealed that PKC activation was blocked after PHA stimulation in Jurkat transfectants expressing the p70 KIR. These observations were confirmed by demonstrating that a recombinant KIR cytoplasmic tail also inhibited the PKCα activation *in vitro*. Taken together, these data strongly suggest that KIR inhibits the T cell AICD by blocking FasL induction upon stimulation, and this process seems to be accomplished by PKC recruitment to the membrane proximal PKC binding site and subsequent inhibition of PKC activation against the activation stimuli.

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국문요약

Inhibitory Killer Immunoglobulin-like Receptor (KIR)에 의한 Jurkat T 세포주의 Activation-Induced Cell Death (AICD) 억제 기전의 규명

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자연살 세포 및 일부 $CD8^+$ T 세포에 표현되는 killer cell immunoglobulin-like receptor (KIR)는 표적 세포의 class I MHC 분자와 접합하여 자연살 세포 또는 T 세포의 활성을 억제하는 수용체이다. KIR 분자를 표현하는 $CD8^+$ T 세포는 체내에서 memory 세포로서, oligoclonal 또는 monoclonal expansion하는 특징을 가진다는 점과, KIR 형질변환 마우스에서 KIR 분자가 $CD8^+$ T 세포의 생존을 도와주어, 결과적으로 T 세포가 활성화 자극 후 소멸하는 과정인 activation-induced cell death (AICD)로부터 T 세포를 보호한다는 최근의 실험 결과는 KIR와 T 세포 AICD의 관련성에 대한 연구의 필요성을 시사한다. 따라서 본 연구에서는 KIR 분자의 표현이 Jurkat T 세포주의 AICD에 미치는 영향과 그 기전을 알아 보고자 하였다. 그 결과, KIR 분자의 하나인 p70 KIR를 과발현시킨 Jurkat 세포 주는 리간드 접촉과 무관하게 phytohemagglutinin (PHA)이나 phorbol ester/ionomycin 자극에 의해 유도된 AICD에 저항성을 보였으며, 그 기전은 T 세포 자극 후 유도되는 FasL 발현의 억제에 의함을 밝혔다. 그리고 Jurkat 세포주의 AICD 억제에 관여하는 KIR 분자의 세포 내 부위인 세포막 인접 20 개의 아미노산에 protein kinase C (PKC)의 기질로 작용할 수 있는 serine기가 존재한다는 점에 착안하여 활성화 자극전후 KIR분자와 PKC의 접합 여부와 KIR 표현이 PKC 활성에 미치는 영향을 보았다. 그 결과, PHA에 의해 유도된 PKCα와 θ의 활성이 KIR 표현 Jurkat T 세포 주에서 현저히 억제되어 있음을 관찰하였다. 또한, 대장균에서 발현된 KIR 세포 내 부위 단백은 *in vitro* PKCα 활성을 효과적으로 억제하였다. 따라서, KIR 분자가 Jurkat 세포주의 AICD 과정을 FasL의 발현 억제를 통해 저지하며, 이 과정은 KIR의 세포막 인접 PKC 부위를 통한 PKC의 직접적인 억제가 관여할 것으로 추정된다. 본 연구는 KIR 분자를 통한 새로운 신호 전달 기전이 존재함을 밝혔으며, 이는 억제성 수용체인 KIR의 면역학적인 역할을 이해하는데 기여할 것으로 생각되며, 정확한 분자 기전을 밝히고자 하는 추가의 연구가 필요할 것으로 사료된다.

핵심되는 말 : KIR, AICD, Jurkat T 세포, PKC, FasL