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Activation of human natural killer cells by the soluble form of cellular prion protein

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ABSTRACT

Cellular prion protein (PrP^C) is widely expressed in various cell types, including cells of the immune system. However, the specific roles of PrP^C in the immune system have not been clearly elucidated. In the present study, we investigated the effects of a soluble form of recombinant PrP^C protein on human natural killer (NK) cells. Recombinant soluble PrP^C protein was generated by fusion of human PrP^C with the Fc portion of human IgG_1 (PrP^C-Fc). PrP^C-Fc binds to the surface of human NK cells, particularly to $CD56^{dim}$ NK cells. PrP^C-Fc induced the production of cytokines and chemokines and the degranulation of granzyme B from NK cells. In addition, PrP^C-Fc facilitated the IL-15-induced proliferation of NK cells. PrP^C-Fc induced phosphorylation of ERK-1/2 and JNK in NK cells, and inhibitors of the ERK or the JNK pathways abrogated PrP^C-Fc-induced cytokine production in NK cells. In conclusion, the soluble form of recombinant PrP^C-Fc protein activates human NK cells via the ERK and JNK signaling pathways.

1. Introduction

The cellular prion protein (PrP^C) is a 32 kDa glycophosphatidylinositol (GPI)-anchored glycoprotein in the lipid raft of the plasma membrane and is widely expressed in neurons and various non-neuronal cell types [1]. PrP^C is also expressed on the surface of many types of immune cells, including T cells, natural killer (NK) cells, monocytes, macrophages and dendritic cells [2,3]. Previous studies reported diverse actions of PrP^C on immune cells [4]. For example, one report demonstrated that incubation of T cells with anti-PrP^C monoclonal antibodies causes crosslinking of cell surface PrP^C proteins and resulted in the internalization of cell surface PrP^C into endosomes, leading to the phosphorylation of ERK-1/2 [5].

Cell surface PrP^C protein can be shed from neuronal cells or lymphocytes into the cell microenvironment or onto other cells in a variety of forms [6]. The shedding of PrP^C protein from cell surface is known to be mediated by metalloproteinase, such as disintegrin and metalloproteases (ADAMs) [7,8]. As a result, soluble PrP^C protein is present in the culture medium of splenocytes and in human serum [2,9]. However, information concerning the effects of soluble PrP^C protein on immune cells is currently limited. A previous study examined the effect of soluble PrP^C protein on monocyte/macrophage cells and found that ERK-1/2 and Akt kinase are activated by soluble PrP^C protein [10]. Recently, we generated a soluble form of recombinant PrP^C protein by fusing it with the Fc portion of human IgG_1 (PrP^C-Fc) and found that it increased phagocytic activity and cytokine production of human monocytes via the ERK and NF-κB pathways [11].

NK cells are an important component of the innate immune system because their effector functions are regulated by a repertoire of germline-encoded receptors that are formed without somatic recombination. In humans, two major subsets of NK cells, $CD56^{dim}$ and $CD56^{bright}$ NK cells, are discriminated by their levels of CD56 expression. Among them, $CD56^{dim}$ NK cells constitute approximately 90% of the NK cells, exhibit a mature phenotype, perform cytolysis, and produce cytokines such as interferon (IFN)-γ [12,13]. Cytotoxicity and cytokine production by NK cells are regulated by various cytokines and NK receptors [14]. Cytokines

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such as IL-2, IL-12, IL-18 and IL-15 potently activate effector functions of NK cells [15]. When NK cells come in contact with target cells, the effector functions of the NK cells are regulated by a balance between signals generated from both activating receptors and inhibitory receptors [16]. When NK cells are activated, the cells degranulate cytotoxic proteins such as perforin and granzymes and secrete IFN-γ, TNF-α, and other cytokines and chemokines.

In the present study, we examined the effects of recombinant soluble PrP^C-Fc protein on human primary NK cells. In particular, we studied its binding to NK cells and the effects on cytokine production, degranulation, and proliferation. Furthermore, we examined which signal pathways are involved in the effects of PrP^C-Fc on human NK cells.

2. Materials and methods

2.1. Isolation of human primary NK cells

Peripheral blood mononuclear cells (PBMCs) from adult healthy donors were isolated using Ficoll–Hypaque density gradients. NK cells were isolated from PBMCs using a MACS Negative Isolation Kit (Miltenyi Biotec, Auburn, CA, USA). Prior to using the isolated NK cells in the experiments, the NK cells were treated with an Fc blocking reagent (Miltenyi).

2.2. Generation and purification of recombinant human PrP^C-Fc

A recombinant soluble human PrP^C protein was generated and purified as previously described [11]. Briefly, a plasmid encoding PrP^C-Fc, composed of amino acids 21–230 of human PrP^C fused with the Fc portion of human IgG1 at the C-terminus, was expressed in HEK293E cells. Culture supernatants from transfected cells were purified using a Protein A-Sepharose column (Amersham Biosciences, Sunnyvale, CA, USA) according to the manufacturer's instructions. The purified recombinant proteins were dialyzed against PBS and analyzed by SDS-PAGE. The endotoxin level was less than 0.01 EU/ml in the recombinant protein.

2.3. Antibodies and reagents

Antibodies that were used for multicolor flow cytometry are as follows: anti-CD3-V500, anti-CD4-PE-Cy7, anti-CD8-APC-H7, anti-IFN-γ-APC, anti-TNF-α-PE-Cy7, and anti-CD107a-PE (all from BD Biosciences, San Jose, CA, USA); and anti-CD56-APC (Miltenyi) and anti-myc-AlexaFluor 488 (from Millipore, Temecula, CA, USA). Antibodies that were used for immunoblotting are as follows: anti-ERK1/2, anti-IKK, anti-JNK, anti-phospho-JNK and anti-phospho-ERK1/2 (all from Cell Signaling Technology, Beverly, MA, USA); and anti-p38, anti-phospho-p38 and anti-GAPDH (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). For some experiments, chemical inhibitors were added to the culture medium 1 h prior to treatment with recombinant proteins. The following chemicals were used in the experiments: SN50, PD98059, MG132 and SP600125 (Calbiochem, San Diego, CA, USA); and wortmannin (Sigma–Aldrich, St. Louis, MO, USA).

2.4. Flow cytometry

PBMCs or isolated NK cells were incubated with an FcR blocking reagent for 30 min at 4 °C prior to staining with fluorochrome-

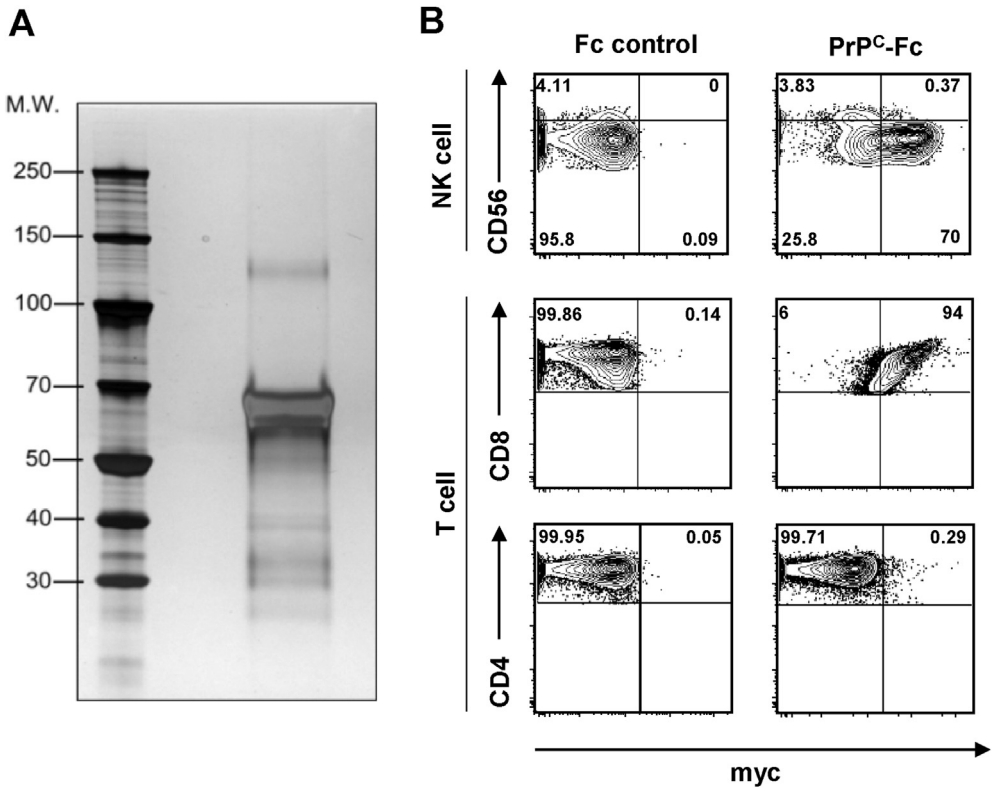

Fig. 1. Binding of recombinant soluble PrP^C-Fc protein to NK cells and $CD8^+$ T cells. (A) Purification of recombinant soluble PrP^C-Fc protein. Silver staining was performed to identify the purified proteins in SDS-PAGE. (B) Binding of recombinant soluble PrP^C-Fc to NK cells and $CD8^+$ T cells. Freshly isolated human PBMCs were incubated with soluble PrP^C-Fc protein (2 μg/ml) or Fc control protein (2 μg/ml). The Fc portion was tagged with myc peptide. Flow cytometry analysis was performed using an anti-myc-AlexaFluor 488 antibody. The data were analyzed in the gates of $CD3^-CD56^+$ NK cells, $CD3^+CD8^+$ T cells, and $CD3^+CD4^+$ T cells. NK cells were further divided to $CD56^{dim}$ NK cells and $CD56^{bright}$ NK cells. The data are representative of three independent experiments.

conjugated antibodies. For intracellular cytokine staining, isolated NK cells were treated with PrP^C-Fc; anti-CD107a-PE and brefeldin A (BD Biosciences) were added 1 h after the PrP^C-Fc treatment, and the culture was maintained for an additional 5 h. Surface staining was performed after staining of dead cells using a Live/Dead Fixable Cell Stain Kit (Invitrogen, Carlsbad, CA, USA). The cells were then fixed, permeabilized, and stained with fluorochrome-conjugated antibodies. Flow cytometry was performed using an LSRII Flow Cytometer (BD Biosciences), and the data were analyzed using FlowJo software (Treestar, San Carlos, CA, USA).

2.5. Quantification of cytokine production

NK cells were stimulated with PrP^C-Fc, culture supernatants were harvested 6 h after the stimulation, and IFN-γ levels were measured using a sandwich ELISA, as previously described [17]. Anti-IFN-γ Ab (Clone 2G1; Thermo Scientific, Rockford, IL, USA) was coated at 0.5 μg/ml in a maxi-soap 96-well plate (Nunc, Roskilde, Denmark), and IFN-γ was detected using a biotin-conjugated polyclonal anti-IFN-γ antibody (Thermo Scientific) and streptavidin-horseradish peroxidase (HRP) (BD Bioscience).

For quantification of IFN-γ, TNF-α, MIP1β, RANTES, IL-2, IL-6, IL10, MCP1α, and granzyme B, a cytometric bead array was used. Briefly, 50 μL of mixed capture beads and 50 μL of each culture supernatant were incubated for 1 h, and 50 μL of mixed PE detection reagents were then added to the bead-sample mixture and incubated for an additional 2 h. An LSR II Flow Cytometer (BD Biosciences) was used to quantify the fluorochrome intensity. The data were analyzed with FlowJo software (TreeStar).

2.6. RNA extraction, cDNA synthesis, and real-time quantitative PCR

NK cells were harvested 8 h after PrP^C-Fc treatment. Total RNA isolation, cDNA synthesis and TaqMan real-time quantitative PCR were performed as previously described [18]. Briefly, total RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA, USA), and

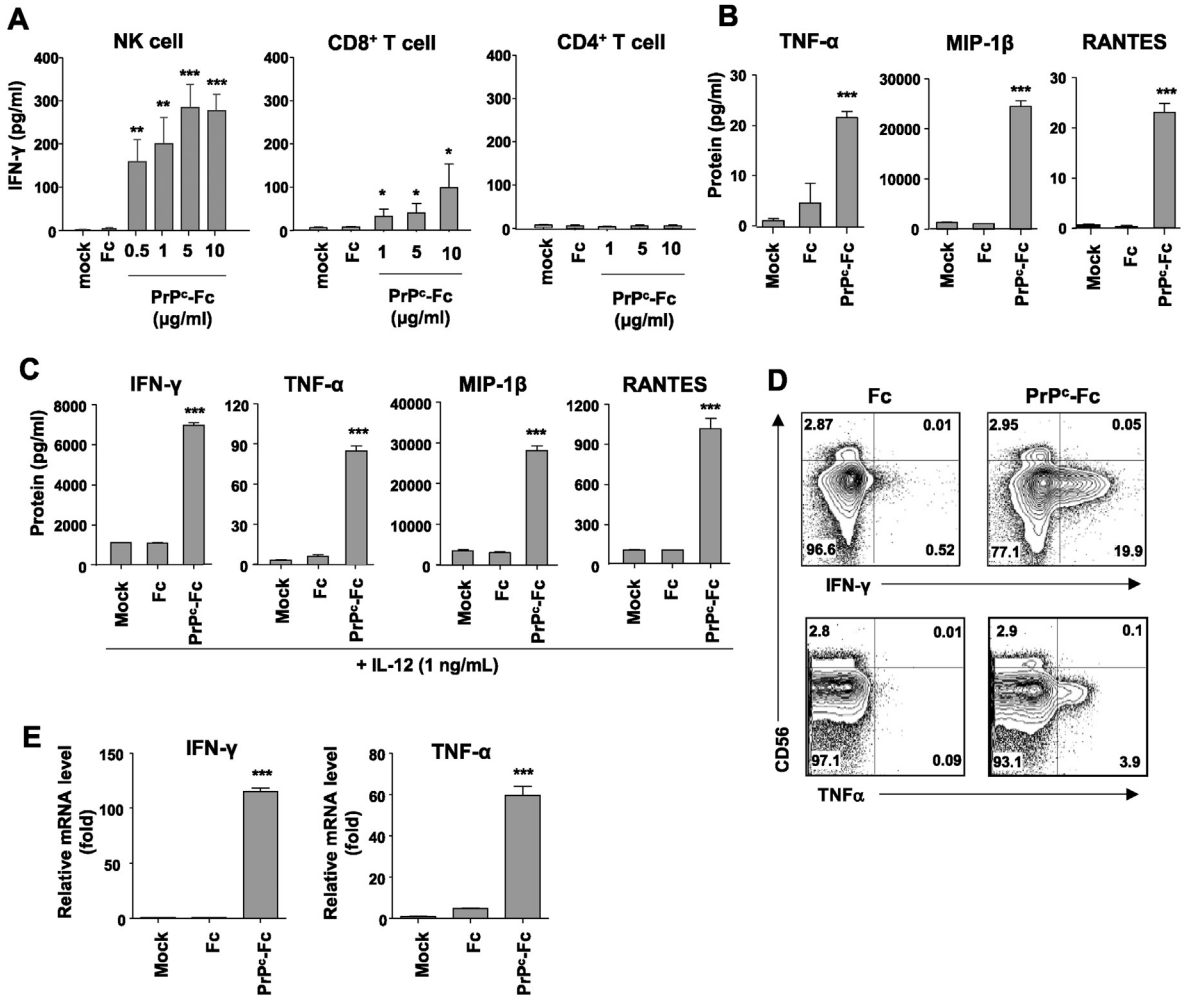

Fig. 2. Production of cytokines and chemokines by NK cells stimulated with soluble PrP^C-Fc. (A) Production of IFN-γ in NK cells and $CD8^+$ T cells after stimulation with soluble PrP^C-Fc in a dose-dependent manner. Cells were incubated with soluble PrP^C-Fc protein (0.5–10 μg/ml) or Fc control protein (10 μg/ml) for 6 h, and secreted IFN-γ was measured using a sandwich ELISA. The data are presented as the mean ± s.d. (n = 5). $^{**}P < 0.01$. $^{*}P < 0.5$. (B) Production of TNF-α, MIP-1β and RANTES in NK cells stimulated with soluble PrP^C-Fc. NK cells were incubated with soluble PrP^C-Fc protein (2 μg/ml) or Fc control protein (2 μg/ml) for 6 h, and secreted cytokines and chemokines were measured using a cytometric bead array. The data are presented as the mean ± s.d. (n = 3). $^{***}P < 0.001$. (C) Production of cytokines and chemokines by combined treatment with soluble PrP^C-Fc and IL-12. NK cells were incubated with IL-12 (1 ng/ml) and soluble PrP^C-Fc protein (2 μg/ml) or Fc control protein (2 μg/ml) for 6 h, and secreted cytokines and chemokines were measured using a cytometric bead array. The data are presented as the mean ± s.d. (n = 3). $^{***}P < 0.001$. (D) Production of cytokines in $CD56^{dim}$ NK cells after stimulation with soluble PrP^C-Fc. NK cells were incubated with soluble PrP^C-Fc protein (2 μg/ml) or Fc control protein (2 μg/ml) for 6 h in the presence of brefeldin A, then permeabilized and stained for IFN-γ and TNF-α. The data are representative of three independent experiments that produced similar results. (E) Increased mRNA level of cytokines in NK cells stimulated with soluble PrP^C-Fc. NK cells were incubated with soluble PrP^C-Fc protein (2 μg/ml) or Fc control protein (2 μg/ml) for 6 h, and real-time qPCR was performed to quantify the mRNA levels of IFN-γ and TNF-α. The data are presented as the mean ± s.e.m. (n = 3). $^{***}P < 0.001$.

first-strand cDNA was synthesized using a High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA). TaqMan Gene Expression Assays (Applied Biosystems) were used to determine the mRNA levels of target genes. The results were standardized to an endogenous control, β-actin.

2.7. Immunoblotting

Immunoblotting was performed as previously described [18]. Briefly, cell lysates were prepared using RIPA buffer, and 10 μg of cell lysate was loaded onto SDS-PAGE gels. After blotting onto a PVDF membrane, the membrane was incubated with primary antibodies overnight at 4 °C, or for 1 h at room temperature. The signal was detected using HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and enhanced chemiluminescence reagents (Thermo Scientific).

2.8. Carboxyfluorescein diacetate (CFSE)-labeled cell proliferation assay

Cell proliferation assays using CFSE were performed as previously described [17]. NK cells were labeled with 5 μM CFSE (Invitrogen) and washed twice with complete medium. CFSE-labeled NK cells were stimulated with 10 ng of IL-15 and 1 μg of PrP^C-Fc. After 6 days, the cells were harvested and flow cytometry was performed using LSR II (BD Bioscience) and FlowJo software (Treestar) to determine the percentage of $CFSE^{low}$ cells.

2.9. Statistical analysis

Most data are presented as the mean ± the standard error of the mean (s.e.m.). Unpaired *t*-tests or two-tailed Mann–Whitney *U*-tests were performed for statistical analysis. All of the analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA). A *P* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Soluble PrP^C-Fc protein binds to $CD56^{dim}$ NK cells and $CD8^+$ T cells

Recombinant soluble PrP^C-Fc was prepared with the construct encoding amino acids 21–230 of human PrP^C fused with the Fc portion of human IgG1. Purified PrP^C-Fc protein was identified by SDS-PAGE analysis (Fig. 1A). The molecular weight of the recombinant PrP^C-Fc was 55–65 kDa under reducing conditions. Next, we examined the binding of PrP^C-Fc to the surface of various types of lymphocytes. PrP^C-Fc protein was tagged with myc at the C-terminus of Fc, to permit the detection of PrP^C-Fc protein bound to the cell surface using an anti-myc antibody. Flow cytometry analysis revealed that PrP^C-Fc bound to NK cells, particularly to $CD56^{dim}$ NK cells (Fig. 1B). PrP^C-Fc also bound to $CD8^+$ T cells, but barely to $CD4^+$ T cells (Fig. 1B).

3.2. Soluble PrP^C-Fc induces the production of cytokines and chemokines in NK cells

We attempted to determine if soluble PrP^C-Fc protein can stimulate NK cells to produce cytokines and chemokines. PrP^C-Fc induced IFN-γ production in NK cells in a dose-dependent manner (Fig. 2A). PrP^C-Fc also induced IFN-γ production in $CD8^+$ T cells, although the level of secreted IFN-γ was much higher in NK cells than in $CD8^+$ T cells (Fig. 2A). In $CD4^+$ T cells, PrP^C-Fc protein did not

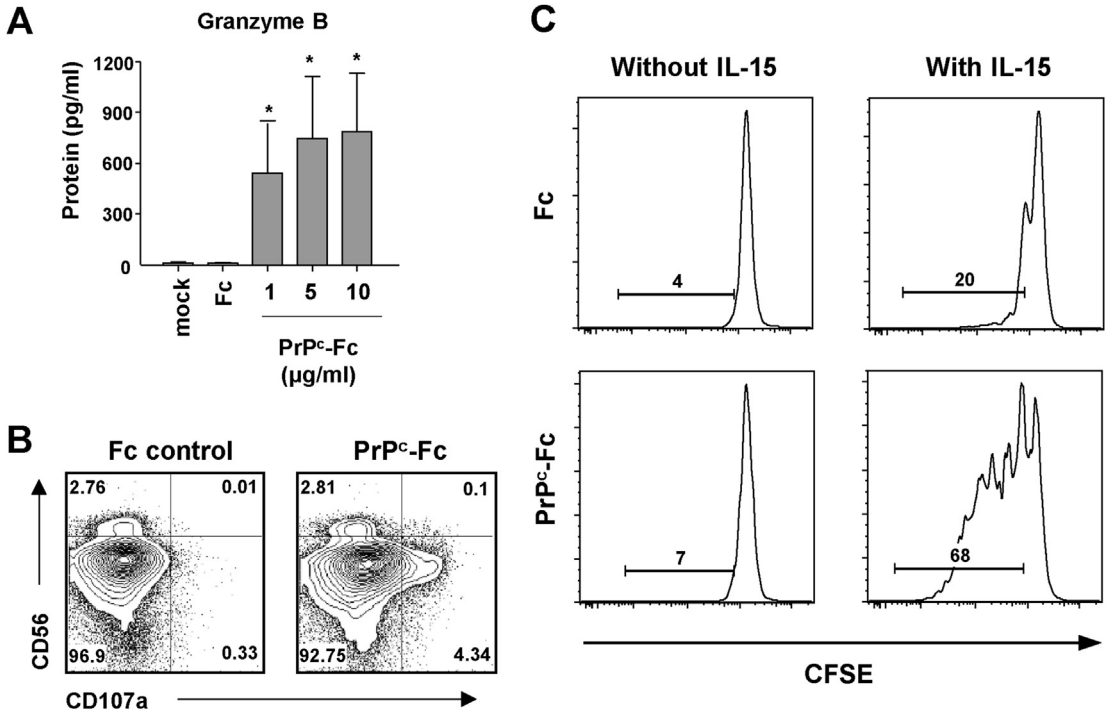

Fig. 3. Degranulation of granzyme B and proliferation of NK cells stimulated with soluble PrP^C-Fc. (A) Degranulation of granzyme B from NK cells by soluble PrP^C-Fc. NK cells were incubated with soluble PrP^C-Fc protein (1–10 μg/ml) or Fc control protein (10 μg/ml) for 6 h, and the concentration of granzyme B was measured in culture supernatants using a cytometric bead array. The data are presented as the mean ± s.d. (n = 5). *$P < 0.05$. (B) CD107a, a degranulation marker, in NK cells after soluble PrP^C-Fc treatment. NK cells were incubated with soluble PrP^C-Fc protein (2 μg/ml) or Fc control protein (2 μg/ml) for 6 h in the presence of anti-CD107a-PE, and flow cytometry analysis was then performed. The data are representative of three independent experiments that produced similar results. (C) Proliferation of NK cells by soluble PrP^C-Fc treatment. CFSE-labeled NK cells were incubated with IL-15 (10 ng/ml) and soluble PrP^C-Fc protein (2 μg/ml) or Fc control protein (2 μg/ml) for 6 days, and flow cytometry analysis was then performed. Percentages of $CFSE^{low}$ cells are indicated in the histograms. Representative data from three independent experiments are shown.

induce the production of IFN-γ (Fig. 2A). We examined the production of other cytokines and chemokines and found that PrP^C-Fc protein also stimulated NK cells to produce TNF-α, MIP-1β and RANTES (Fig. 2B). When treated with PrP^C-Fc and IL-12 together, NK cells produced more cytokines and chemokines than when treated IL-12 alone (Fig. 2C), which suggests that PrP^C-Fc protein also enhances IL-12-induced production of cytokines and chemokines in NK cells. Intracellular cytokine staining demonstrated that PrP^C-Fc induced IFN-γ and TNF-α production in $CD56^{dim}$ NK cells, rather than in $CD56^{bright}$ NK cells (Fig. 2D). The mRNA levels for IFN-γ and TNF-α were increased after stimulation of NK cells with PrP^C-Fc (Fig. 2E), indicating that PrP^C-Fc induces IFN-γ and TNF-α production in NK cells at the transcriptional level.

3.3. Soluble PrP^C-Fc enhances degranulation of granzyme B and proliferation of NK cells

The amount of degranulated granzyme B, a cytotoxic protein, was measured after the stimulation of NK cells with PrP^C-Fc, and it was observed that PrP^C-Fc increased the level of degranulated granzyme B from NK cells in a dose-dependent manner (Fig. 3A). We also measured the level of CD107a (also known as lysosomal-associated membrane protein-1) that was exposed on the surface of NK cells and bound to extracellular anti-CD107a antibodies during the stimulation with PrP^C-Fc protein. In this assay, $CD107a^+$ cells are considered to degranulate in response to stimulation of the cells [19,20]. The percentage of $CD107a^+$ cells was increased after stimulation with PrP^C-Fc, particularly in $CD56^{dim}$ NK cells (Fig. 3B). To determine whether the proliferation of NK cells is also increased by PrP^C-Fc treatment, NK cells were stimulated by PrP^C-Fc protein and IL-15, and CFSE dilution proliferation assays were performed. Proliferation of NK cells was increased by IL-15 treatment and was further increased by combined treatment with IL-15 and PrP^C-Fc, although PrP^C-Fc alone did not induce NK cell proliferation (Fig. 3C).

3.4. Soluble PrP^C-Fc activates NK cells via ERK-1/2 and JNK pathways

To elucidate the signal pathways that are involved in PrP^C-Fc protein-induced NK cell activation, we studied several signal

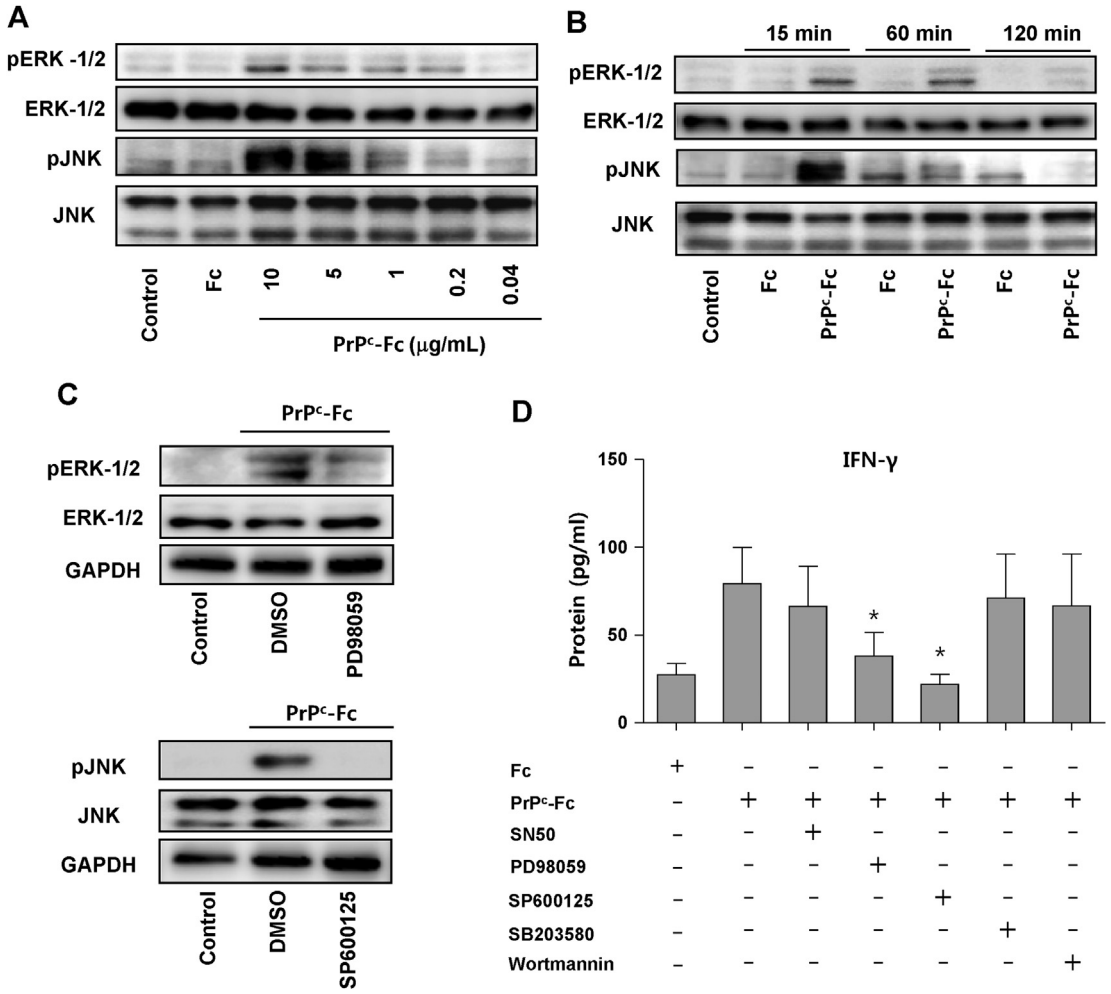

Fig. 4. ERK-1/2 and JNK activation in NK cells stimulated with soluble PrP^C-Fc. (A, B) Phosphorylation of ERK-1/2 and JNK in NK cells stimulated with soluble PrP^C-Fc. NK cells were incubated with soluble PrP^C-Fc protein (doses are indicated) or Fc control protein (10 μg/ml) for 15 min (A) or with soluble PrP^C-Fc protein (5 μg/ml) or Fc control protein (5 μg/ml) for the indicated times (B). Immunoblotting was performed to identify the phosphorylation of ERK-1/2 and JNK. Representative results from four independent experiments are shown. (C) Effects of PD98059 (ERK inhibitor) and SP600125 (JNK inhibitor) on the phosphorylation of ERK and JNK in PrP^C-Fc-treated NK cells. NK cells were pretreated with PD98059 (20 μM) or SP600125 (50 μM) for 1 h and then treated with PrP^C-Fc (5 μg/ml) for 15 min. Representative results from two independent experiments are shown. (D) Effect of inhibitors of various signal pathways on PrP^C-Fc-induced IFN-γ production in NK cells. NK cells were pretreated with inhibitors for 1 h and were then treated with PrP^C-Fc (5 μg/ml) for 6 h. The concentrations of inhibitors are as follows: SN50 (NF-κB inhibitor; 10 μM), PD98059 (20 μM), SP600125 (50 μM), SB203580 (p38 inhibitor; 10 μM) and wortmannin (PI3K inhibitor; 1 μM). A sandwich ELISA was performed to detect secreted IFN-γ. The data are presented as the mean ± s.d. (n = 3). $^*P < 0.05$.

pathways, including the ERK-1/2 and JNK pathways. After treatment of NK cells with PrP^C-Fc protein, ERK-1/2 and JNK were phosphorylated in a dose-dependent manner (Fig. 4A). The phosphorylation of ERK-1/2 and JNK was observed 15–60 min after treatment with PrP^C-Fc (Fig. 4B). However, we did not observe the phosphorylation of other signal molecules such as p38 and IKK after treatment with PrP^C-Fc (data not shown). PD98059 (ERK inhibitor) and SP600125 (JNK inhibitor) abrogated the phosphorylation of ERK-1/2 and JNK in PrP^C-Fc-treated NK cells (Fig. 4C) and attenuated the production of IFN-γ in PrP^C-Fc-treated NK cells (Fig. 4D). However, SN50 (NF-κB inhibitor), SB203580 (p38 inhibitor) and wortmannin (PI3K inhibitor) did not attenuate IFN-γ production in PrP^C-Fc-treated NK cells. Collectively, these data suggest that the ERK-1/2 and JNK pathways are involved in PrP^C-Fc protein-induced NK cell activation.

4. Discussion

In this study, we have demonstrated that recombinant soluble PrP^C-Fc activates human NK cells to produce cytokines via the ERK-1/2 and JNK pathways. It is well known that the mitogen activated protein kinase (MAPK) and phosphatidylinositide 3-kinases (PI3K) pathways are involved in NK cell activation [21–24]. In the present study, the JNK pathway was strongly activated by PrP^C-Fc treatment of NK cells, and the ERK-1/2 pathway was also activated. Moreover, inhibition of JNK or ERK-1/2 attenuated IFN-γ production in PrP^C-Fc-treated NK cells. However, inhibition of p38 MAPK and PI3K had no significant effect on IFN-γ production in PrP^C-Fc-treated NK cells. A previous report showed that a PrP^C-Fc fusion protein induced phosphorylation of ERK and PI3K in a macrophage-like cell line [10]. In addition, a recent study from our group showed that PrP^C-Fc activates monocytes via the ERK-1/2 and NF-κB signaling pathways [11]. The discrepancies of signaling pathways involved in the immune cell activation by soluble PrP^C-Fc protein may arise from differences in cell types, particularly the difference between monocyte/macrophage cells and NK cells.

Although various molecules have been identified as binding partners with PrP^C [25], it remains to be elucidated which surface receptor of NK cells is responsible for the binding of soluble PrP^C-Fc protein to NK cells. Initially, we hypothesized that cell surface PrP^C protein itself may serve as a binding partner for recombinant soluble PrP^C-Fc protein. Previous studies showed that treatment with peptides that bind to cell surface PrP^C activates the ERK pathway in various cell lines, including the lymphoid BW5147 cell line, the Jurkat T cell line, and the mouse neurohypothalamic GTI-7 cell line [5,26,27]. These reports led us to hypothesize that cell surface PrP^C might be a putative binding partner for recombinant soluble PrP^C-Fc protein. However, knock-down studies of cell surface PrP^C protein in NK cells did not decrease the binding of soluble PrP^C-Fc protein and PrP^C-Fc-induced IFN-γ production (data not shown), implying that cell surface PrP^C protein does not serve as a binding partner for soluble PrP^C-Fc protein. Next, we assumed that NK-activating receptors may serve as binding partners for soluble PrP^C-Fc protein. However, blocking antibodies to various NK-activating receptors (NKG2D, NCRs and DNAM1) did not have any significant effect on the binding of soluble PrP^C-Fc to the NK cell surface, or to PrP^C-Fc-induced IFN-γ production (data not shown).

In the present study, we demonstrated that recombinant soluble PrP^C-Fc protein activates human NK cells, leading to increased cytokine secretion and proliferation. PrP^C-Fc-induced NK cell activation occurs via the ERK-1/2 and JNK pathways. Additional studies are required in order to elucidate the NK cell surface binding partner of soluble PrP^C-Fc protein and the exact mechanisms involved in PrP^C-Fc-induced NK cell activation.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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