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The role of IL-18 in the modulation of matrix metalloproteinases and migration of human natural killer (NK) cells

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Abstract **In this study, we examined whether interleukin-18 (IL-18) affects natural killer (NK) cells' migration and matrix metalloproteinases (MMPs) production. We demonstrated that chemotaxis of human NK cells through basement membrane-like Matrigel was augmented by IL-18. As well, IL-18 stimulation induces the production of activated forms of matrix metalloproteinase-2 (MMP-2) as well as the production of pro-MMP-2 from NK cells. We also demonstrated that MT1-MMP expression on human NK cells, which is a major activator of MMP-2, was induced by IL-18 stimulation coordinated with MMP-2 activation. These data suggest that the MT1-MMP/MMP-2 system participates in the degradation of basement membrane components and thus contributes to NK cell migration.**
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Keywords: Interleukin-18; Matrix metalloproteinase; Membrane-type matrix metalloproteinase; NK cell

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc endo-proteinases that are a secreted or membrane-bound form of protein [1,2]. They play important roles in many biological processes including angiogenesis, inflammation and cancer metastasis [3–5]. The main characteristics of MMPs is the degradation of the extracellular matrix of basement membranes, thus enabling cells to invade into tissues [6–8]. For example, T cells secrete the gelatinases, MMP-2 and MMP-9 in response to cytokines and inflammatory mediators and migrate across basement membrane by degrading extracellular matrix (ECM) using gelatinases [9–11].

Natural killer (NK) cells are endowed with the ability to express spontaneous cytotoxicity against tumor cells or virus-infected cells [12]. Upon several biological responses such as virus infection and carcinogenesis, NK cells encompass movements directed against target cells [13–15]. They should possess proteinases which mediate their transmigration through the ECM component for their migration to the target cells [16–18]. Recent studies have suggested that matrix metalloproteinases play an important role in basement membrane degradation and the transmigration of NK cells [19]. The 92 kDa gelatinase (gelatinase B, MMP-9) and the 72 kDa gelatinase (gelatinase A, MMP-2), which efficiently degrade native collagen type 4 and 5, are believed to play an important role in lymphocyte migration [20]. Recently, membrane-type MMP (MT-MMP) has been shown to be a potent activator of MMP-2 by cleaving the propeptide domain [21,22]. It has also been demonstrated that IL-2 activated NK cells produce MMP-2 and MMP-9 and express MT-MMP [23,24]. Therefore, IL-2 augments NK cells' migration as well as NK cells' cytotoxicity. IL-18 is also essential for NK cells' cytotoxicity and antitumor activity [25,26]. It is probable that IL-18 augments NK cells' migratory ability by producing MMPs in addition to its cytotoxicity. In order to identify the contribution of IL-18 on NK cells' migratory function, we examined the MMPs and MT-MMPs produced by IL-18-stimulated human NK cells.

2. Materials and methods

2.1. Reagents

Recombinant human IL-18 was purchased from MBL (Nagoya, Japan). Endotoxin levels were less than 0.1 ng per 1 mg recombinant IL-18 protein. Recombinant human IL-12 was purchased from Genzyme (Cambridge, MA). Recombinant human IL-2 was kindly provided from Shionogi Pharmaceutical Co. (Osaka, Japan). Anti-human IL-18 neutralizing monoclonal antibodies (125-2H) were purchased from MBL. Anti-human IL-2 neutralizing monoclonal antibodies (5334.21) were purchased from Genzyme. Anti-human IFN-γ neutralizing monoclonal antibodies (NIB42) were purchased from eBioscience (San Diego, USA).

2.2. Isolation of NK cells

NK cells were purified from buffy coat cells (kindly provided by the Nagasaki Red Cross Blood Center, Nagasaki, Japan) isolated from peripheral blood, as previously described [27]. Briefly, peripheral blood mononuclear cells (PBMC) were separated from whole blood by centrifugation over Ficoll–Hypaque. For NK cell isolation, cells were depleted of T lymphocytes, B lymphocytes, and macrophages/monocytes using a NK cell isolation kit (Minitenyi Biotec, Bergisch Galdbach, Germany), including bead-coupled mAb against CD3, CD4, CD19, and CD33. Using a MACS magnetic separator (Miltenyi Biotec), NK cells were purified phenotypically >95% $CD16^{+}$ $CD56^{+}$, as determined by flow cytometry. Freshly isolated NK cells were cultured with serum-free media (RPMI 1640), with or without IL-18, at a concentration of 1×10^{6} cells/ml in 24-well plates (Costar, Cambrige, MA).

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Abbreviations: IL-18, interleukin-18; MMPs, matrix metalloproteinases; MT-MMP, membrane-type matrix metalloproteinase; NK cells, natural killer cells; TIMP, tissue inhibitor of matrix metalloproteinase

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2.3. Migration assay

Cells migration was quantified using Transwell inserts (6.5 μm; Costar, Cambridge, MA) fitted with polycarbonate filters (8-μm pre size; Nuclepore Corp., Pleasanton, CA). The upper sides of the filter were coated with Matrigel (200 $\mu g/cm^2$, Collaborative Research Inc, Bedford, MA) and dried overnight. NK cells (5×10^6 in 1000 μl medium) were added to the upper compartment of the insert with or without IL-18. The lower compartment contained 1500 μl of medium containing identical concentrations of IL-18. The chambers were incubated at 37 °C for 24 h. Then, the numbers of NK cells that had migrated into the lower chamber were counted.

2.4. Gelatin zymography

Culture media were incubated at 37 °C for 20 min in sodium dodecyl sulfate (SDS) sample buffer free of reducing agents and then electrophoresed on 8% polyacrylamide gels containing 0.5% gelatin at 4 °C. After electrophoresis, the gels were washed in 2.5% Triton-X 100 to remove SDS and incubated with 50 mM Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3 for 16 h at 37 °C, and then stained with 0.1% Coomassie Blue R250. The gelatinolytic bands were analyzed by densitometer (Photometrics, Tucson, Arizona). Images from densitometer were transferred to a personal computer and analyzed using IPLab Gel software (Signal Analytics Corporation, Vienna, VA).

2.5. Immunoblot analysis

The expression of MT-MMP on NK cells and the secretions of tissue inhibitor of MMPs (TIMPs) from NK cells were analyzed by immunoblot. For this purpose, cells were washed with cold PBS and lysed by the addition of a lysis buffer containing 1% Nonidet P-40, 50 mM Tris (pH 7.5), 100 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 20 min at 4 °C. Insoluble material was removed by centrifugation at $\times 1500\ g$ for 15 min at 4 °C. The supernatant was saved and the protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). An identical amount of protein (30 μg) from each lysate or culture supernatant was subjected to 10% SDS–polyacrylamide gel electrophoresis. The fractionated proteins were transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL), and the filters were blocked for 1.5 h using non-fat dried milk in Tris-buffered saline (TBS: 50 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.1% Tween 20, washed with TBS, and incubated at room temperature for 2 h at a 1:150 dilution of mouse anti-MT1, 2, 3-MMP or TIMP-1, 2 monoclonal antibodies (Fuji Chemicals, Takaoka, Japan). The membranes were further incubated with a 1:2000 dilution of horseradish peroxidase-conjugated donkey anti-mouse immunoglobulin G (IgG) antibody (Promega, Madison, WI) for 20 min. An enhanced chemiluminescence (ECL) system (Amersham) was used for detection. The filters were subsequently exposed to film for 15 s and the latter was processed.

2.6. RNA preparation and RT-PCR assay

Total cellular RNA was extracted from NK cells using guanidium thiocyanate and phenol (RNAzol B, Cinna/Biotek Labs Int. Inc., Friendswood, TX). First-strand cDNA was synthesized by reverse transcription at 45 °C for 45 min in a 50 μl reaction mixture containing 1 μg of total RNA and MuLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD). After denaturing at 99 °C for 5 min followed by cooling at 5 °C, the cDNA was amplified using PCR. Two microliters of denatured cDNA was amplified by a 20 μl final volume containing 1 U *Taq* DNA polymerase (Gibco-BRL), 1 μM of each primer, Taq polymerase buffer, 1.5 mM $MgCl_2$, and 1.5 mM of each dNTP. PCR was performed in a thermal cycler (Perkin–Elmer Cetus, Foster City, CA) using a program of 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min with a final 10 min extension at 72 °C. The amplified products were subjected to electrophoresis on 2% agarose gel.

The following specific primers were used for MT1-MMP:
5′-AGGCGCCCCCGATGTGGTGTT-3′ (forward),
5′-TGGCCGAGGGGTCACTGGAATGCT-3′ (reverse).
Predicted size of the fragment is 502 bp (corresponding base pairs: 265–766).

For β-actin:
5′-GACGAGGCCCAGAGCAAGAGAG-3′ (forward),
5′-ACGTACATGGCTGGGGTGTTG-3′ (reverse).
Predicted size of the fragment is 284 bp.

3. Results

3.1. Migration of IL-18-stimulated human NK cells

We used a matrigel invasion assay to determine the ability of IL-18-stimulated human NK cells to invade through a model basement membrane. Freshly isolated human NK cells were placed in a Matrigel invasion chamber in the presence or absence of IL-18 for 24 h. As shown in Fig. 1A, IL-18 enhanced NK cell mobility significantly at respective concentrations of 0–100 ng/ml. IL-18 may have a capacity to induce IL-2, which has been shown to augment NK cell migration [11]. Therefore, we examined the effects of anti-IL-2 antibodies on IL-18-stimulated NK cell migration. As shown in Fig. 1B, anti-IL-2 antibodies did not affect IL-18-stimulated NK cell migratory activity. IL-12 is a proinflammatory cytokine produced by macrophages and augments NK cell cytotoxicity [28]. We investigated whether IL-12 induces NK cell migration. As shown in Fig. 1C, IL-12 induces NK cell migration and its migration-inducing activity is similar to those of IL-18. IL-12 has been shown to enhance IL-18-mediated biological activity by inducing IL-18R expression [29]. Therefore, we examined the combined effects of IL-12 and IL-18 on NK cell migration. The combined treatments of IL-18 plus IL-12 upregulates NK cell migratory activity (Fig. 1C).

3.2. Gelatin zymographic analysis of NK cell culture supernatants

We sought to determine whether or not IL-18 enhances the MMP secretion necessary to allow NK cells to migrate across the Matrigel mode basement membrane. Supernatants isolated from IL-18-stimulated NK cells grown in serum-free media were analyzed using SDS–PAGE gelatin zymography. The results indicated two major gelatin-cleaving activities that correspond to the 72-kDa MMP-2 and 92-kDa MMP-9. These two gelatinolytic bands were increased by IL-18 stimulation (Fig. 2A). Gelatinases are known to be secreted in a latent form and converted to the active form through the cleavage of the N-terminal pro-peptide domain by membrane-type matrix metalloproteinase [21,22]. The enzymatic ability of gelatinases is influenced by these activation processes. In gelatin zymography of IL-18-stimulated NK cell culture supernatants, a detectable gelatinolytic band was also noted at about 66 kDa in addition to the 72 kDa MMP-2. This lower product of 66 kDa corresponds to the active form of MMP-2. It is possible that IL-18 stimulates other cytokines, such as IL-2, production from NK cells. As shown in Fig. 2B, IL-2 stimulated the gelatinases secretion from NK cells. To determine whether NK cells secreted gelatinases in response to IL-18 or indirectly via IL-2, IL-2 was blocked by anti-IL-2 neutralizing antibodies. Although anti-IL-18 neutralizing antibodies blocked the IL-18-induced secretion of activated MMP-2, anti-IL-2 neutralizing antibodies did not affect this secretion from NK cells (Fig. 2B and C). IL-18 also induces IFN-γ production from NK cells [30]. To determine whether IL-18-mediated IFN-γ production affects the MMPs secretion from NK cells, we examined the effects of anti-IFN-antibodies on IL-18-induced activated MMP-2 secretion. As shown in Fig. 2D, anti-IFN-γ neutralizing antibodies did not affect IL-18-induced activated MMP-2 secretion from NK cell.

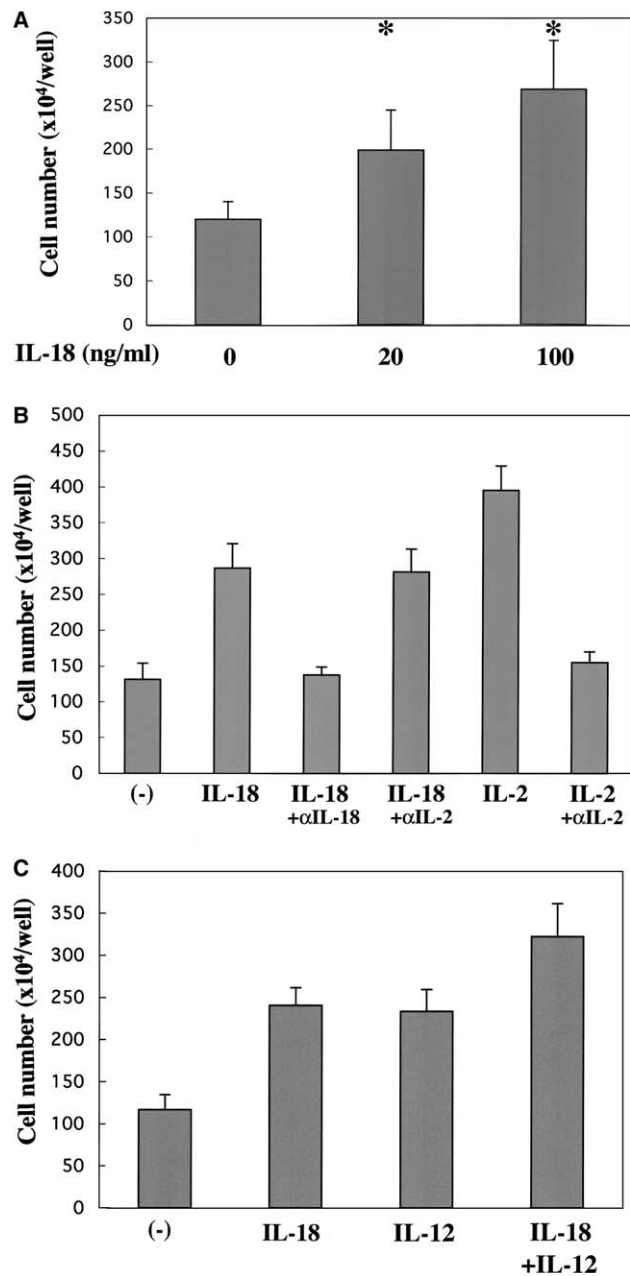

Fig. 1. (A) Effects of IL-18 on human NK cell migration across a model of basement membrane. Human NK cells (5×10^6) in 1.0 ml were placed in the top well of Matrigel invasion chambers with various concentrations of IL-18 for 24 h. Results are expressed as cell numbers invaded into bottom chambers through Matrigels. Data for bar graphs represent means ± S.E.M. of three independent experiments. Statistical significance was performed by Student's t-test. $^*P < 0.05$ versus control (untreated NK cells). (B) Effects of anti-cytokine antibodies on NK cell migration across a model of basement membrane. Human NK cells (5×10^6) in 1.0 ml were placed in the top well of Matrigel invasion chambers with IL-18 (100 ng/ml) in the presence or absence of anti-IL-2 (αIL-2 final concentrations; 2 μg/ml) or anti-IL-18 (αIL-18 final concentrations; 10 μg/ml) neutralizing antibodies for 24 h. Results are expressed as cell numbers invaded into bottom chambers through Matrigels. Data for bar graphs represent means ± S.E.M. of two independent experiments. (C) Combined effects of IL-12 and IL-18 on NK cell migration across a model of basement membrane. Human NK cells (5×10^6) in 1.0 ml were placed in the top well of Matrigel invasion chambers with IL-18 (100 ng/ml) and IL-12 (10 ng/ml) for 24 h. Results are expressed as cell numbers invaded into bottom chambers through Matrigels. Data for bar graphs represent means ± S.E.M. of two independent experiments.

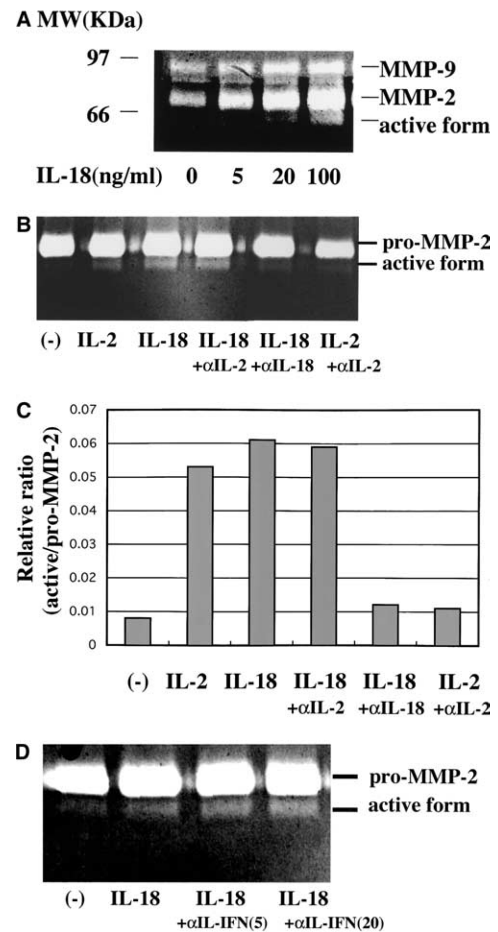

Fig. 2. (A) IL-18-induced gelatinase secretion from human NK cells. Human NK cells were cultured with IL-18 in serum-free culture media for 24 h. Conditioned media were analyzed by gelatin zymography. Note the activated MMP-2 with lower molecular weights in addition to 72 kDa MMP-2 in IL-18-stimulated NK cells-conditioned media. The data shown are representatives of three independent experiments. (B,C) Effects of anti-cytokine antibodies on gelatinases secretion from NK cells. (B) Human NK cells were cultured with IL-18 (100 ng/ml) or IL-2 (100 IU/ml) in the presence or absence of anti-IL-2 (αIL-2) or anti-IL-18 (αIL-18) neutralizing antibodies for 24 h. Conditioned media were analyzed by gelatin zymography. Note the activated MMP-2 with lower molecular weights in addition to 72 kDa MMP-2 in IL-18-stimulated NK cells-conditioned media. The data shown are representatives of two independent experiments. (C) Densitometric analyses of B are presented as the relative ratio of active form/pro-form of MMP-2. (D) Effects of anti-IFN-γ antibodies on gelatinases secretion from NK cells. Human NK cells were cultured with IL-18 (100 ng/ml) in the presence or absence of anti-IFN-γ neutralizing antibodies (final concentrations; 2 μg/ml, 10 μg/ml) for 24 h. Conditioned media were analyzed by gelatin zymography. Note the activated MMP-2 with lower molecular weights in addition to 72 kDa MMP-2 in IL-18-stimulated NK cells-conditioned media. The data shown are representatives of two independent experiments.

We analyzed the presence of other MMPs in NK-cell conditioned media. However, we could not detect MMP-3 and MMP-13 in NK cell conditioned media with or without IL-18 by immunoblot analysis (data not shown).

3.3. Expression of MT-MMP

MT-MMP, which is expressed on the cell surface, has the ability to convert the latent form of MMP-2 to the active form

[21]. In order to confirm the expression of MT1-MMP on NK cells, we performed immunoblot analysis using a specific antibody. Cellular lysates prepared from IL-18-stimulated or unstimulated NK cells were analyzed by immunoblot using anti-MT1-MMP monoclonal antibodies. As shown in Fig. 3A, the expression of MT1-MMP was faintly detected in unstimulated NK cells. As shown in Fig. 3A, IL-18 stimulation induced the expression of MT1-MMP in NK cells in a dose-dependent manner. This IL-18 induced MT1-MMP expression on NK cells was completely inhibited by anti-IL-18 antibodies (Fig. 3B). Although MT2-MMP was also detected in NK cells, its expression was not affected by IL-18 stimulation (Fig. 3C). MT3-MMP was not detected in unstimulated or IL-18-stimulated NK cells (data not shown). We also tried to determine whether IL-18 treatment induces MT1-MMP mRNA expression in human NK cells using RT-PCR analysis. MT1-MMP and β-actin cDNA were amplified by PCR. MT1-MMP mRNA expression was induced in IL-18-treated NK cells but not in untreated NK cells. No change was noted in the amplified fragments of the housekeeping gene β-actin (Fig. 4).

3.4. TIMPs secretion from IL-18-treated NK cells

The enzymatic activities of gelatinases are also influenced by a family of TIMPs. Therefore, we analyzed the secretion of TIMPs from IL-18-stimulated NK cells using immunoblot analysis. Although TIMP-1 and TIMP-2 proteins were identified in NK cells-conditoned media, these secretions were not altered with or without IL-18 stimulation (data not shown).

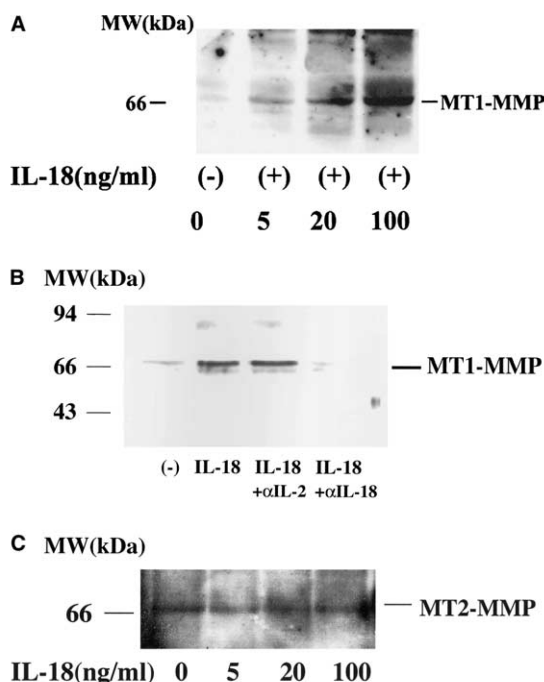

Fig. 3. (A,B) Immunoblot analysis of MT1-MMP in IL-18-treated human NK cells. Human NK cells were cultured with IL-18 in the presence (B) or absence (A) of anti-IL-18 neutralizing antibodies for 24 h. Blots of SDS–PAGE gels of IL-18-treated NK cell lysates were probed with antibodies to human MT1-MMP. The data shown are representatives of three independent experiments. (C) Immunoblot analysis of MT2-MMP in IL-18-treated human NK cells. Human NK cells were cultured with IL-18 for 24 h. Blots of SDS–PAGE gels of IL-18-treated NK cell lysates were probed with antibodies to human MT2-MMP. The data shown are representatives of two independent experiments.

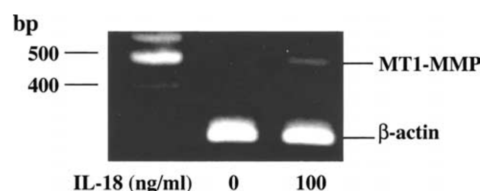

Fig. 4. RT-PCR analysis of mRNA for MT1-MMP in NK cells. Human NK cells were treated with or without IL-18 (100 ng/ml) for 12 h. Total RNA was reverse transcribed following PCR amplification with primer for MT1-MMP and β-actin. Ethidium bromide staining of PCR products. The data shown are representatives of three independent experiments.

4. Discussion

IL-18, originally designated as IFN-γ inducing factor, is a cytokine produced by activated macrophages, monocytes, and dendritic cells during innate immune response [31,32]. IL-18 has been shown to have the capacity to amplify IFN-γ secretion by NK and NKT cells [25,26]. Although NK cell numbers are normal in IL-$18^{-/-}$ mice, NK cell function including cytotoxicity has been shown to be significantly reduced [33]. Collectively, these data demonstrated that IL-18 is essential for NK cell function. We sought to determine whether IL-18 enhances NK cell cytotoxicity by inducing transmigration or interaction with ECM. IL-18 stimulation significantly augments the migration of human NK cells through a layer of Matrigel by degrading the constituents of the Matrigel.

The migration of immune cells including T cells has been previously shown to be mediated by MMPs [34]. MMPs are members of a family of Zn^{2+}-dependent endopeptidases and exhibit full activity for a wide range of connective matrix proteins [2]. In gelatin zymography, the active form of MMP-2 as well as pro-MMP-2 was detected in IL-18-stimulated NK cell culture media. MMPs activity is induced by a coordinated increase in transcription, secretion and proteolytic activation [35]. During the proteolytic process, latent pro-MMPs are activated by cleavage of the pro-peptide domain and conformational change of the Zn^{2+}-binding motif [36]. Various factors including serine proteinases are known to activate pro-MMP. However, pro-MMP-2 is unique in that it is not activated by serine proteinases, but is activated by MT-MMP [22]. MT1-MMP has been identified as a putative cell membrane-associated activator of pro-MMP-2 and shown to be expressed in IL-2-activated NK cells [24].

One of the interesting findings of the present study is that pro-MMP-2 is secreted as an active form in IL-18-stimulated NK cells, suggesting that pro-MMP-2 is activated on NK cells. Murine IL-2-activated NK cells was shown to express MT1-MMP by immunoblot analysis [24]. Therefore, we investigated the expression of MT-MMP in IL-18-stimulated NK cells. Our data clearly indicated that IL-18 induced MT1-MMP expression on human NK cells. These data suggest that IL-18-stimulated NK cells not only secrete pro-MMP-2 but also efficiently convert pro-MMP-2 to an active form by expressing

MT1-MMP on its surface. Because MT1-MMP, MT2-MMP, and MT3-MMP are believed to be tissue activators of pro-MMP-2 [37], we examined their protein expression on NK cells by immunoblot analysis. Although MT1-MMP is induced in IL-18-stimulated NK cells, MT2-MMP, which is constitutively expressed in NK cells, was not increased by IL-18. The activation activity of MT3-MMP is much weaker than that of MT1-MMP [38]. The increase in MT1-MMP protein expression in NK cells was much larger than that expected from mRNA. Upregulation of MT1-MMP protein level in IL-18-treated NK cells occurred at least at the pre-translational level as shown by the increased MT1-MMP mRNA. However, additional post-translational regulation cannot be ruled out, since post-translational upregulation mechanism of MT1-MMP protein has been described [39].

This is the first report demonstrating that IL-18 clearly induced MMP-2 secretion and MT1-MMP expression using freshly isolated human NK cells. These data suggest that both gelatinases and MT1-MMP might be biologically important in the penetration of NK cells through the basal lamina. In summary, we showed that IL-18 potentiated NK cells' migratory ability by inducing MMP-2 production but also by promoting MMP-2 activation, probably by inducing MT1-MMP expression. This IL-18-mediated modulation of MMP-2 and MT1-MMP suggests that this cytokine may play a potential role in NK cell migration.

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