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PRSS14/Epithin is induced in macrophages by the IFN-γ/JAK/STAT pathway and mediates transendothelial migration

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ABSTRACT

PRSS14/Epithin (also known as matriptase and ST14), a member of the type II transmembrane serine proteases, is primarily found in a subpopulation of normal epithelial cells and in epithelial cancers. Its known functions include maintaining the epithelial barrier, thymic development, and cancer progression. In this study, we show that several macrophage cell lines and activated bone marrow-derived macrophages also express PRSS14/Epithin. Surface expression, as well as cytoplasmic expression, was detectable upon activation by IFN-γ, but not TNF-α or TGF-β. Induction of the protein appeared to be restricted to macrophages. IFN-γ showed a biphasic regulation in RAW264.7 cells, and upregulated expression was sustained for several days. This induction by IFN-γ was partially through the increase of PRSS14/Epithin mRNA production, which is downstream of the JAK pathway, shown by the inhibition by tyrphostin AG490. Using chromatin immunoprecipitation, we verified that two sites among six putative STAT1 binding sites in the PRSS14/Epithin promoter were occupied by STAT1 upon activation. Treatment with IFN-γ enhanced the serum-triggered transendothelial migration of RAW264.7 cells, but not that of PRSS14/Epithin knock-down RAW264.7 cells, although they express multiple markers such as ICAM1, CD80, and CD40 at normal levels. These data strongly suggest that PRSS14/Epithin plays an important role in the transendothelial migration of activated macrophages in the inflammatory microenvironment, and the mode of action is similar to the events in cancer metastasis.

1. Introduction

Monocytes/macrophages have various functions in multiple tissues. Inflammatory signals usually cause the migration of these cells to inflammatory sites, including migration through endothelial cells [1]. They respond to and are activated by a variety of molecules, including IFN-γ [2]. INF-γ is important in activation of macrophages and in diverse cellular effects, including antigen processing and presentation, antiviral and antimicrobial activities, antiproliferative effects, apoptosis, modulation of immune system, and trafficking, which are frequently accompanied by an upregulation or downregulation of gene expression.

PRSS14/Epithin is a type II membrane serine protease [3]. Because PRSS14/Epithin is normally expressed in epithelial tissues and overexpressed in malignant epithelial tumor cells, studies on PRSS14/Epithin have been focused on its function in normal epithelia and epithelial tumors [4]. PRSS14/Epithin is indispensable for normal development of the skin to maintain epithelial barrier function. More recent studies showed that the function of PRSS14/Epithin is not restricted to epithelial development. Knock-out of PRSS14/Epithin results in defects in hair follicle development and thymocyte survival. In contrast, overexpression of PRSS14/Epithin is related to the development of various epithelial cancers [5–8]. Transgenic mice that overexpress PRSS14/Epithin in skin tissue showed spontaneous development of squamous carcinoma in a Ras-dependent and independent manner [9]. In addition, expression of PRSS14/Epithin is directly and indirectly involved in metastasis through enhanced transendothelial migration [10].

Several studies, including our own, demonstrated that angiogenesis and metastasis during cancer progression were also directly affected by PRSS14/Epithin [10–12]. We showed that several tumor cells shed PRSS14/Epithin into media, and soluble PRSS14/Epithin induced the migration, rearrangement, and differentiation of endothelial cells through unknown target molecules. Moreover, it was proposed that PRSS14/Epithin promotes cancer

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metastasis [6]. Recently, we reported that PRSS14/Epithin is critical for the epithelial-to-mesenchymal transition [12] and transendothelial migration of epithelial tumor cells through the cleavage of Tie2 in endothelial cells [10].

In this study, we demonstrate the role of PRSS14/Epithin in macrophages. We show that macrophages express PRSS14/Epithin upon IFN-γ activation, and the JAK/STAT1 pathway is involved in its induction of expression. In addition, PRSS14/Epithin plays an important role in IFN-γ-enhanced transendothelial migration of macrophages.

2. Materials and methods

2.1. Cell culture and reagents

RAW264.7 cells were grown in DMEM with 10% fetal Bovine Serum (FBS). WEHI-3 cells were grown in IMDM with 10% FBS and 0.05% β-mercaptoethanol. Bone marrow-derived macrophages (BMDMs) were prepared from femurs of C57BL/6 mice and grown in DMEM containing 10% FBS and 10% L929-conditioned media for 5 days. 427.1.86 and 4T1 cells were maintained in DMEM with 10% FBS. To generate R-KD and R-Con cell lines, a target GGTGCGCTT CAAACTCTTC and its scrambled sequence GTGCGCGTTACAATCTCTC were cloned into pSUPER (OligoEngine). RAW264.7 cells cotransfected with pEGFP-C1 (Clontech) were selected with 1 mg/ml G418. AG490, JNK inhibitor II, PD98059 and SB203580 were from Calbiochem; Interferon-γ, TNF-α, and TGF-β were from Invitrogen/GibcoBRL.

2.2. Immunoblotting

Cell lysates were harvested with SDS-containing sample buffer with 2-mercaptoethanol, subjected to SDS–PAGE, and transferred onto nitrocellulose membrane (Whatman). Immunoblots were developed using enhanced chemiluminescence.

2.3. Immunocytochemistry

RAW264.7 cells grown on 0.1% gelatin-coated coverslips were fixed with 3.7% formaldehyde for 10 min. The samples were permeabilized with PBS containing 0.5% Triton X-100, followed by blocking with 0.1% gelatin and 10% goat serum in PBS, and were stained with anti-N55 antibody.

2.4. Flow cytometry

For PRSS14/Epithin, sheep anti-matriptase (R&D) was used as the primary antibody with anti-sheep antibody conjugated with phycoerythrin. For other markers, monoclonal antibodies against CD40, CD54, and CD80 were used [13].

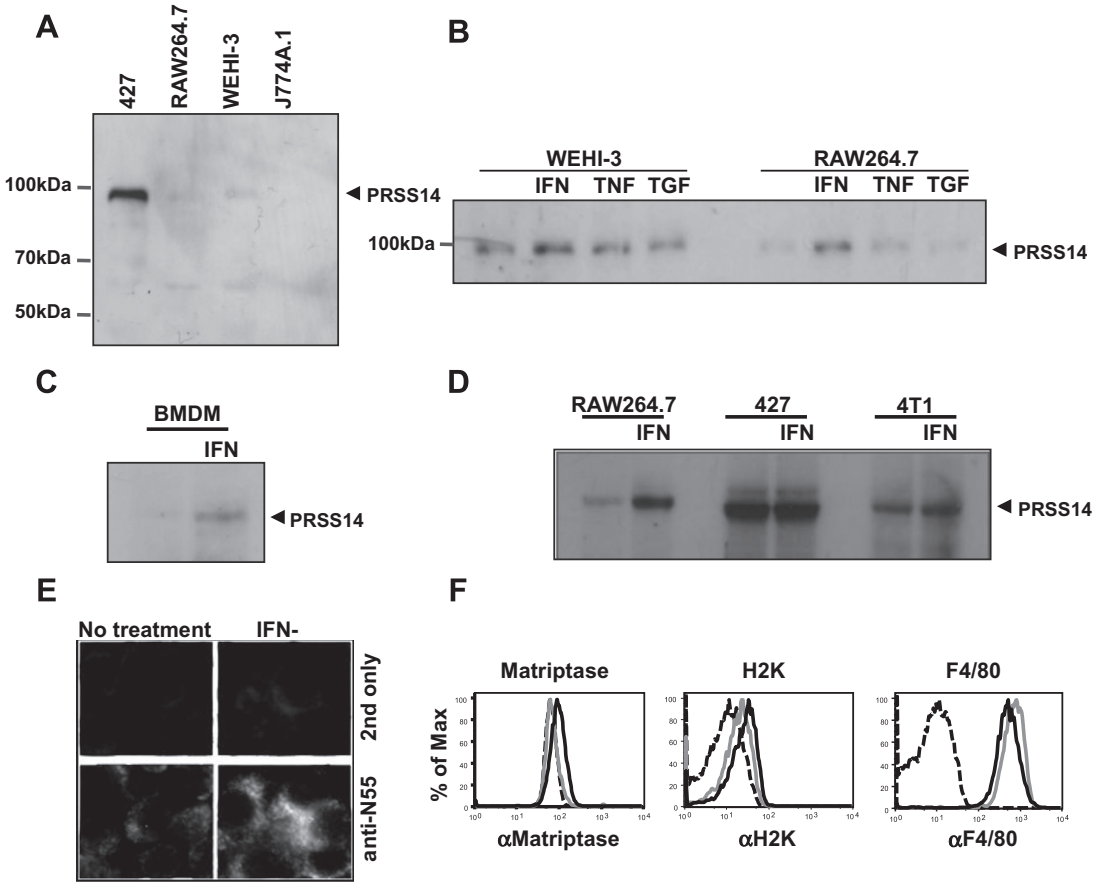

Fig. 1. PRSS14/Epithin expression is induced in activated macrophages. (A) RAW264.7 and WEHI-3 macrophage cell lines and 427.1.86 epithelial cells express PRSS14/Epithin. The levels of PRSS14/Epithin were analyzed by Western blotting using the monoclonal antibody mAb5. (B) IFN-γ (300 unit/ml), but neither TNF-α (25 ng/ml) nor TGF-β (5 ng/ml), increased the expression .of PRSS14/Epithin in WEHI-3 and RAW264.7 cells. Cells were treated with all cytokines for 16 h. (C) BMDMs were treated with 300 unit/ml IFN-γ for 16 h. (D) Expression of PRSS14/Epithin in other epithelial cancer cell lines was not elevated by IFN-γ. (E) Enhanced signals of PRSS14/Epithin were shown in IFN-γ-treated RAW264.7 cells. (F) Surface expression of PRSS14/Epithin, H2K, and F4/80 detected by flow cytometry. Dark line, activated RAW264.7; gray line, unactivated cells; dotted line, negative staining control. Matriptase, human ortholog of PRSS14/Epithin; H2K, major histocompatibility antigen class I; F4/80, macrophage marker.

2.5. Quantitative real-time PCR

Total RNAs were extracted with the RNeasy Kit (Qiagen). Reverse transcription of RNAs was performed with Superscript III (Invitrogen), and the cDNAs were subjected to quantitative real-time PCR on an Applied Biosystems 7300 (Applied Biosystems). Primers for PRSS14/Epithin are 5′-TCATCGCCTACTACTGGTCAGAGT-3′, 5′-TGGCGCGATCAACCTCTT-3′ and 5′-(FAM)-CAGCATCCCCCCACACCTGGC-(TAMRA)-3′ and for GAPDH are 5′-TGGCCTCCAAGGAGTAAGAAAC-3′, 5′-GGGATAGGGCCTCTCTTGCT-3′ and 5′-(FAM)-CTGGACCACCCACCCCAGCAA-(TAMRA)-3′.

2.6. Chromatin immunoprecipitation

Chromatin immunoprecipitation assay (ChIP) was performed as previously described [14]. Three micrograms of anti-STAT1 SM1 antibody was used for each ChIP assay. Primers for amplification of six GAS elements are indicated in Table 1.

2.7. Transendothelial migration assay

Procedures were essentially the same as described previously [10], except the incubation time. In our experiments, RAW264.7 cells were incubated for 48 h in Boyden chambers.

3. Results

3.1. PRSS14/Epithin is induced by interferon-γ in macrophages

It was reported that human monocytes/macrophages express MT-SP1, another name for PRSS14/Epithin [15]. Here, we observed that PRSS14/Epithin is expressed in WEHI-3 and RAW264.7 mouse macrophage lines, although their expression is far less than 427.1.86 thymomas (Fig. 1A). To examine the changes of its expression during activation, several known activators were tested in WEHI3 and RAW264.7 cells. IFN-γ and TNF-α are known for the classical M1 type activation of macrophages, and TGF-β for alternative M2 type activation [16,17]. The treatment with IFN-γ, but not TNF-α or TGF-β, for 16 h increased the expression of PRSS14/Epithin in WEHI3 and RAW264.7 cells (Fig. 1B). Furthermore, LPS, S1P, IL-4, IL-10, and MSP failed to induce expression of PRSS14/Epithin (data not shown). To examine whether this effect of IFN-γ was an *in vitro* cell artifact, we prepared fresh BMDM and tested for induction. As expected, BMDM showed induction of PRSS14/Epithin expression in response to IFN-γ (Fig. 1C). We also tested whether epithelial 427.1.86 and 4T1 cells respond to IFN-γ by inducing PRSS14/Epithin expression. 427.1.86 cells constitutively expressed PRSS14/Epithin at high levels and did not show any further increase, while 4T1 cells showed a slightly increased level upon IFN-γ treatment (Fig. 1D). However, they were still capable of STAT1 phosphorylation with IFN-γ treatment (data now shown). These results suggest that the induction of PRSS14/Epithin by IFN-γ is macrophage-specific.

When cellular localization was tested by immunocytochemistry using the antibody against the cytoplasmic portion of PRSS14/Epithin (N55), it was found that the protein appeared dispersed in activated RAW264.7 cells (Fig. 1E). It did not accumulate at cell–cell contact regions as found in the case of epithelial cell types [18,19]. This suggests that PRSS14/Epithin in macrophages has a different mode of action. Surface expression of the protein was also analyzed by flow cytometry using anti-human matriptase antibody. As seen in Fig. 1F, RAW264.7 cells expressed PRSS14/Epithin on the surface only when they were activated. Staining levels in the unactivated cells overlapped with the staining of cells treated only with secondary antibodies. Activation by IFN-γ was verified by the increase of the MHCI molecule, H2-K. However, IFN-γ did not alter the levels of the macrophage marker F4/80, indicating that the increased staining of PRSS14/Epithin was not an activation artifact.

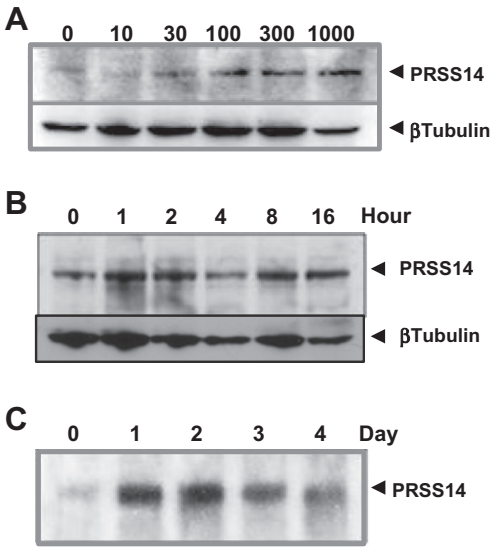

Fig. 2. IFN-γ dose- and treatment time-dependent expression of PRSS14/Epithin in RAW264.7 cells. (A and B) Expression of PRSS14/Epithin was increased by IFN-γ in a dose (unit/ml) and time-dependent manner. For the dose-dependent experiment, IFN-γ was treated for 16 h at the indicated dose. For the time-dependent experiment, 300 unit/ml of IFN-γ was used. (C) Enhanced expression of PRSS14/Epithin lasted for at least 4 days. All the results were obtained by immunoblotting with mAb5.

Next, we tested the kinetic profiles of PRSS14/Epithin expression. The levels of PRSS14/Epithin were increased as concentrations of IFN-γ were increased, up to 300 unit/ml (Fig. 2A). Expression of PRSS14/Epithin showed a biphasic pattern with the peaks at 1–2 and 16 h (Fig. 2B). The late response to IFN-γ lasted for several days (Fig. 2C). These data indicate that PRSS14/Epithin protein may be involved in at least two types of physiological processes in macrophages.

3.2. IFN-γ increases mRNA levels of PRSS14/Epithin through the JAK/STAT1 pathway

In order to investigate the mechanism of PRSS14/Epithin upregulation, the levels of PRSS14/Epithin mRNA were analyzed by quantitative real-time PCR. RAW264.7 and BMDM cells were treated with IFN-γ for 16 h and examined for the levels of mRNA. Results showed that the steady state levels of PRSS14/Epithin mRNA were increased about twofold upon activation (Fig. 3A and B). To verify the transcriptional activation, a luciferase assay using the PRSS14/Epithin promoter was performed. Matrix analysis of sequences from the mouse and human promoter of PRSS14/Epithin showed high evolutionary conservation between the two species in a 4 kb region (data not shown); therefore, the DNA fragment of this region was cloned into a vector for use in luciferase assays (Fig. 3C).

Table 1
Primers for ChIP assay.

GAS	Primer sequences (5′-3′)
G1	TAGCCAATGGGGTGTAGACTCTAT, GTGCACCCACAGTGTGTCTG
G2	GCTCTGTCGATGGAATGCTGAC, CACTAATCAATAGCCTGACGGTTC
G3	GAACAAGGCATCGACTACACTCT, CTCCTGACTCTCAAACTGCTGT
G4	ACAGCAGTTTGAGAGTCAGGAG, CACCTTGTCAAGCCTATTTGTCTC
G5	GGGGTCTGTAACTGATACGTTCTC, GTCTAACTCAGGCTAAATCACCTG
G6	GCCTCAAGGACATGAGTGAGTT, AGGCTAACTCAGAACCAAATGAGC

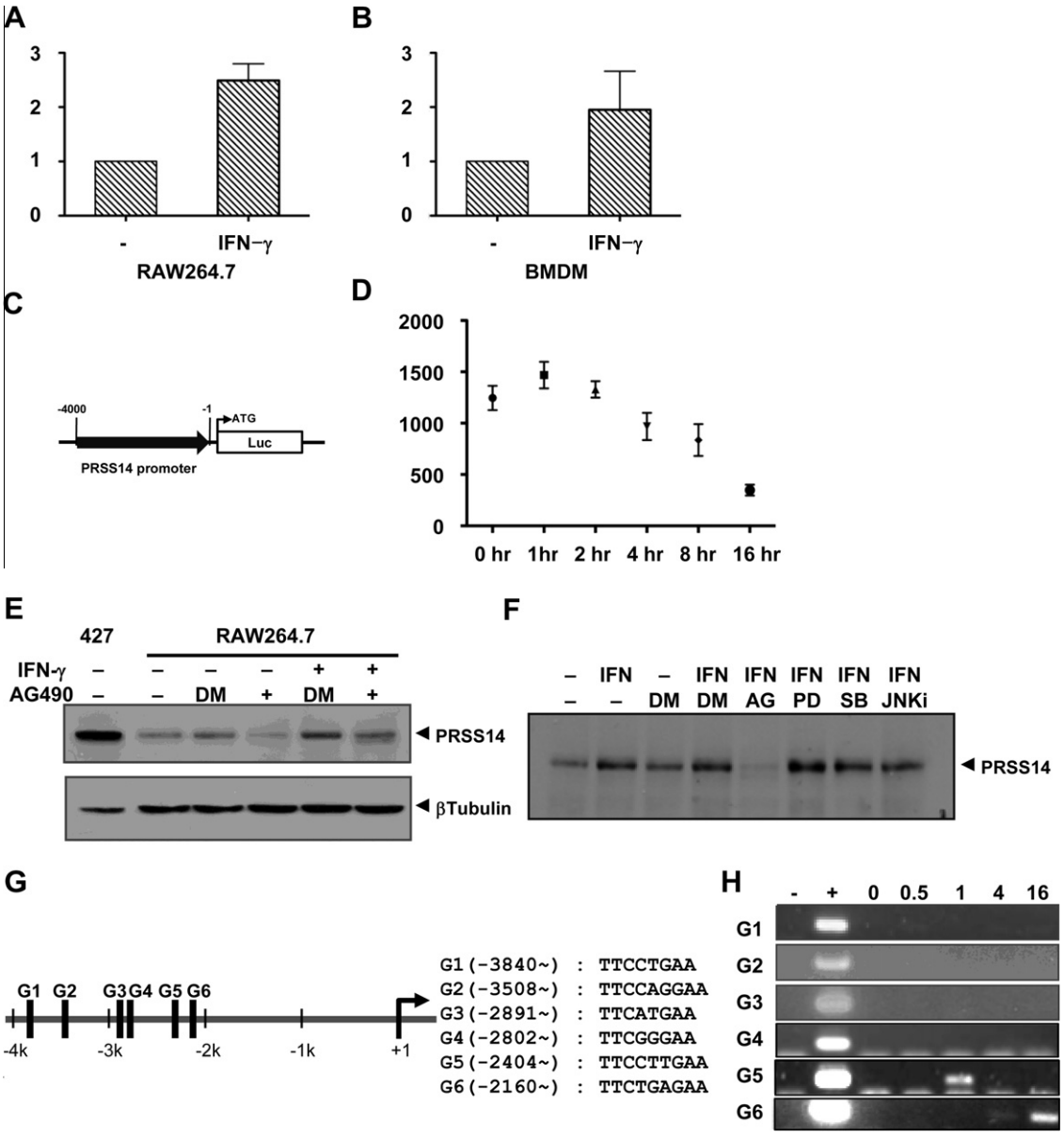

Fig. 3. IFN-γ regulates PRSS14/Epithin mRNA in RAW264.7 cells and BMDMs, and the JAK/STAT signaling pathway is involved in the regulation of PRSS14/Epithin expression. (A and B) Quantitative Real-time PCR showed that IFN-γ (300 unit/ml, 16 h) elevates the level of PRSS14/Epithin mRNA of RAW264.7 cells and bone marrow-derived macrophages. All data were normalized to the signal level of GAPDH mRNA. Data represent means ± standard error of two independent experiments. (C and D) For the luciferase assay, 4 kb of the promoter sequence was cloned into the pGL3 vector (Promega). Data represent means ± standard error from two independent experiments. (E) Pretreatment with the JAK inhibitor AG490 (30 mM) reduced PRSS14/Epithin expression in RAW264.7 cells. DMSO and AG490 in DMSO were added for 1 h before the treatment with IFN-γ for 16 h. (F) AG490 (30 μM), PD98059 (40 μM), SB203580 (5 μM) and JNK II inhibitor (2 μM) were added for 1 h before treatment with IFN-γ. (G) Six putative GAS elements (G1, G2, G3, G4, G5, and G6) were found within 4 kb upstream of the promoter sequence. Each sequence element and its location are indicated. (H) ChIP assay and semi-quantitative RT-PCR were used for STAT1 binding of putative GAS elements. IFN-γ (300 unit/ml) were treated for the indicated times.

With a peak at 1 h after IFN-γ treatment, a slight increase and a significant decline in luciferase activity were observed, suggesting that PRSS14/Epithin transcription is involved only in the early activation phase, and other mechanisms and/or regulatory elements are involved in later stages (Fig. 3D).

IFN-γ is well-known for regulating the transcription of many genes through the JAK/STAT pathway and alternative signaling pathways [20]. First, we tested whether JAK mediates IFN-γ-dependent PRSS14/Epithin gene expression. To inhibit the IFN-γ-dependent JAK, we treated cells with 30 μM of tyrphostin AG490, an inhibitor of JAK2, for 1 h before the treatment of IFN-γ. AG490 reduced the levels of PRSS14/Epithin in IFN-γ-treated RAW264.7 cells (Fig. 3E). These data suggest that the activation of JAK2 by IFN-γ is necessary to induce PRSS14/Epithin expression. In contrast, pretreatment of MAPK inhibitors, PD98059 (MEK inhibitor), SB203580 (p38 inhibitor), or JNK inhibitor II did not inhibit the expression, indicating that alternative pathways by IFN-γ are not involved in the induction of PRSS14/Epithin in RAW264.7 cells (Fig. 3F).

In the 4-kb region of the PRSS14/Epithin promoter, there are six putative IFN-γ activation site (GAS) elements (TTCN(2-4)GAA) (Fig. 3G), which were originally identified for binding to a dimer of STAT1 phosphorylated by JAK [21,22]. To identify which GAS elements are functional, we examined STAT1 binding to these putative GAS sequence elements by chromatin immunoprecipitation. The chromatin fragments from RAW264.7 cells treated with

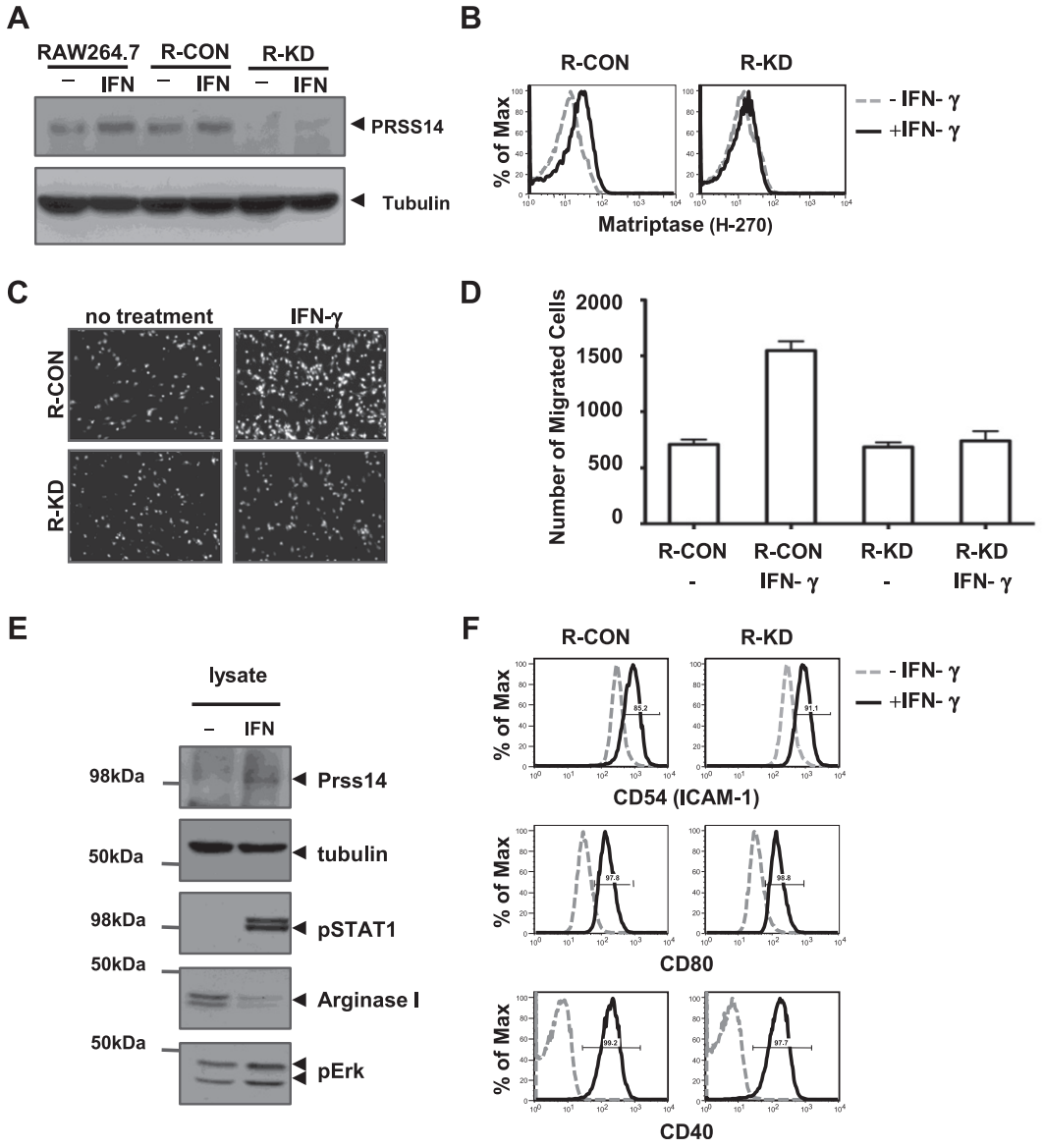

Fig. 4. Role of PRSS14/Epithin in the transendothelial migration of activated macrophages. (A) The expression of PRSS14/Epithin and tubulin is shown for knockdown of PRSS14/Epithin and the parent cell and control lines. Representative knockdown R-KD showed low levels of PRSS14/Epithin expression regardless of IFN-γ treatment. R-Con was used as control. (B) Flow cytometric analysis of surface PRSS14/Epithin expression using anti-human matriptase. R-cont and R-KD were used in the analysis. (C) Representative images for migrated cells are shown. (D) Migrated cells were counted using ImageJ (National Institute of Health). Graph represents means ± standard error from each of five images. (E) Immunoblotting of RAW264.7 cells with or without activation of PRSS14/Epithin (Prss14), tubulin (tubulin), phospho-STAT1 (pSTAT1), arginase I (Arginase I) and phospho-ERK (pERK). (F) Analysis of surface markers in R-cont and R-KD RAW264.7 cells by flow cytometry. Live cell gates were analyzed for the expression of CD54, CD80, and CD40.

IFN-γ for various time periods were immunoprecipitated with an anti-STAT1 SM1 antibody and amplified by PCR against each putative GAS element. Among these, the fifth and sixth putative GAS elements (G5, G6) were clearly complexed with STAT1 at different time points, G5 in the early (1 h) response period and G6 in the late (16 h) response period (Fig. 3H). Therefore, we conclude that STAT1 binds to the GAS elements of the promoter and is included in the transcription complex for the production of PRSS14/Epithin mRNA.

3.3. PRSS14/Epithin is critical for the transendothelial migration of activated macrophages

Membrane type serine proteases or secreted proteases can cleave the extracellular matrix (ECM) and enhance the mobility of cells through the ECM or endothelial cells [23,24]. Moreover, PRSS14/Epithin has critical roles in transendothelial migration of epithelial cancer cells *in vitro* and in cancer metastasis [10]. Because the migration of activated macrophages is essential to their functions, we investigated the effects of enhanced expression of PRSS14/Epithin in transendothelial migration of macrophages. We generated PRSS14/Epithin knock-down RAW264.7 (R-KD) cells and control cell lines (R-Con). While PRSS14/Epithin in R-Con cells showed IFN-γ-dependent induction, that of R-KD cells was hardly detectable in the cell lysate or on the cell surface, regardless of IFN-γ treatment (Fig. 4A and B).

To investigate the ability of these cells to migrate through endothelial cells, we measured the transendothelial migration of the cells through an MS1 mouse endothelial cell layer in Boyden chambers. After 2 days, when the MS1 monolayer was confluent,

RAW264.7 cells were added into the inner chamber, and the number of cells that migrated through the MS1 monolayer into the outer chamber was counted. R-Con RAW264.7 cells showed enhanced transendothelial migration in response to IFN-γ (Fig. 4C and D) to the same extent of original RAW264.7 cells (data now shown). However, R-KD cells did not show such an enhancement in response to IFN-γ (Fig. 4C and D). The numbers of migrated cells increased by about 200% with IFN-γ treatment (Fig. 4D). These data show that IFN-γ enhanced the transendothelial migration of macrophages by inducing the expression of PRSS14/Epithin.

In order to verify the activation type and status of RAW264.7 cells after IFN-γ induction in our system, we investigated the expression of selected molecules that can indicate the types of macrophages (Fig. 4E). Arginase I, an M2 type marker, disappeared after treatment with IFN-γ, indicating that the activation in the IFN-γ system led cells to differentiate to the classical M1 type, as expected. We then tested whether the ablation of PRSS14/Epithin expression altered the fate of macrophage activation using R-KD RAW264.7 cells. As seen in Fig. 4F, the levels of the adhesion molecule ICAM, costimulatory molecule, CD80 and CD40 in R-KD RAW264.7 cells were similar to those of the R-Con RAW264.7 cells, indicating that other activation markers and their functions remained unchanged.

4. Discussion

This study shows that PRSS14/Epithin has roles in macrophages in addition to its role in epithelial and tumor cells. IFN-γ increases the level of PRSS14/Epithin mRNA and protein in macrophages, and the well-known IFN-γ downstream molecules JAK and STAT1 were involved in this process (Figs. 1–3). Moreover, induction of PRSS14/Epithin by IFN-γ is critical for the migration of macrophages through endothelial cells (Fig. 4).

4.1. PRSS14/Epithin expression during macrophage activation

PRSS14/Epithin is expressed in peritoneal macrophages, but not in bone marrow macrophages [15]. It has been shown that peritoneal macrophages express at least tenfold greater PRSS14/Epithin mRNA than that of BMDMs. However, it has not been shown how the mRNA of PRSS14/Epithin is induced in peritoneal macrophages, and if there is any regulatory mechanism for PRSS14/Epithin expression during macrophage differentiation or activation. In this study, we show that the level of PRSS14/Epithin expression in BMDMs can be increased by IFN-γ treatment and that the JAK/STAT pathway is involved in this process (Figs. 1 and 4). Alternative activation signaling is not involved in the induction of PRSS14/Epithin. Induction of PRSS14/Epithin expression is regulated at the transcriptional and post-transcriptional level (Figs. 2 and 3). The discrepancy between the amounts and profiles of steady state levels of mRNA and the levels of luciferase activities indicate that there are strong post-transcriptional mechanism(s) that control the late phase expression of PRSS14/Epithin. Possible candidate mechanisms will be at the regulation of message stability and the translational process. In addition to the regulation mentioned above, other mechanisms in the levels of protein expression can also be achieved at the levels of protein processing and stability. Our result showed that the shedding of PRSS14/Epithin from the membrane in macrophages was also affected by IFN-γ (unpublished result). The detailed mechanisms and their significance in the development and function of macrophages remain to be elucidated.

IFN-γ is a well-known cytokine that activates macrophages and induces their differentiation into the M1 type [17]. Our current study shows that M1 type differentiation of macrophages induces PRSS14/Epithin expression as one of the effector molecules. However, in peritoneal macrophages, PRSS14/Epithin cleaves and activates pro-MSP-1 to MSP-1, which is sufficient for the activation of its receptor RON and downstream signaling [25]. MSP-1 is also capable of differentiating these cells to M2 type macrophages. Therefore, different types of inflammatory signals can lead to the transcription of PRSS14/Epithin.

IFN-γ activates an antimicrobial M1 type macrophage, but MSP-1 alternatively activates M2 macrophages that function in tissue regeneration and immunomodulation, as seen in tumor-associated macrophages (TAMs) [15,26]. We speculate that PRSS14/Epithin can participate in the function of TAM because the activation of MSP-1 and overexpression of PRSS14/Epithin is highly coordinated with cancer metastasis and poor prognosis. Among alternative macrophage activators such as IL-4, IL-10, and TGF-β, none could increase the level of PRSS14/Epithin under our experimental conditions (Fig 1C and unpublished data). However, these results cannot exclude the possibility of its action *in vivo,* where the situation is more complex.

Induction of PRSS14/Epithin gene expression by the IFN-γ/JAK/STAT1 pathway may represent a macrophage-specific mechanism because epithelial cancer cell lines do not have a similar induction profile, but reveal the constitutive expression of the protein (Fig. 1D). This could be achieved by macrophage-specific transacting factors, in addition to the action of STAT1 on the PRSS14/Epithin promoter. Moreover, it was reported that the inhibitor HAI-1 is barely detected in macrophages [15]. Therefore, it is possible that IFN-γ treatment is sufficient for the activation of PRSS14/Epithin in macrophages without the translocation of the protein into cell contact areas. This lack of translocation can explain the dispersed distribution of the protein in activated macrophages.

4.2. Mode of transendothelial migration of macrophages is similar to that of cancer metastasis

Our data show that the migration of activated macrophages through an endothelial layer depends on the induction of PRSS14/Epithin (Fig. 4). When PRSS14/Epithin message was ablated, activated macrophages could not efficiently transmigrate.

Recently, we showed that PRSS14/Epithin regulates an endothelial cell-specific signaling molecule Tie2 during transendothelial migration [10]. PRSS14/Epithin interacts with Tie2, which has roles in the regulation of vessel remodeling and endothelial permeability. PRSS14/Epithin cleaves the extracellular portion of Tie2, which interacts with its ligands, angiopoietin 1 and angiopoietin 2. The cleavage of Tie2 leads to its tyrosine phosphorylation in the cytoplasmic portion in a ligand-independent manner. Phosphorylation of Tie2 recruits several molecules and transduces downstream signaling [27]. Thus, it is highly likely that PRSS14/Epithin in activated macrophages behaves in a similar fashion for transendothelial migration through Tie2 degradation. It is possible that the process of monocyte/macrophage movement to the infected tissues through endothelial cells uses the same mechanism as the extravasation of cancer cells.

Acknowledgments

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