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# 종양미세환경에서 골수유래면역억제세포를 조절하는 GRP78에 의한 전이성 암 진행

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2018년 02월

서울대학교 대학원
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A thesis of the Master's degree

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College of Medicine
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by Yeonju Park

A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Master of Science in Medicine at Seoul National University College of Medicine

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지도교수 이 동 섭

이 논문을 의학 석사 학위논문으로 제출함

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2018년 01월

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# ABSTRACT

# The 78-kDa glucose-regulated protein (GRP78) accentuates the metastatic cancer progression via the modulation of myeloid-derived suppressor cells in cancer microenvironment

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Cancer cells create a cancer microenvironment for cancer growth and survival, and the interaction between cancer cells and cancer microenvionrment plays an important role in multiple aspects of cancer cells growth, invasion, metastasis, and the immune escape by the secretion of a variety of factors.

We previously confirmed that metastatic murine breast cancer cells with high interleukin-6 (IL-6) expression recruited myeloid-derived suppressor cells

(MDSCs) to the cancer site and the other metastatic organs, and recruited MDSCs induce metastasis to distant site through IL-6 trans-signaling. Cancer-derived IL-6 can recruit MDSCs to the cancer microenvironment of non-metastatic breast cancer cells, but it cannot induce MDSC-meditated metastasis. Therefore, I examined the additional cancer-derived factors that can activate MDSCs in the cancer microenvironment to induce metastasis to distant site.

In this study, I characterized cancer-derived the 78 kDa glucose-regulated protein (GRP78) as additional MDSC-activating factors. Metastatic cancer cells expressed high levels of GRP78, and secreted GRP78 protein into the cancer microenvironment. Cancer-derived soluble GRP78 was accentuates the metastatic cancer progression via the modulation of MDSCs in cancer microenvironement. GRP78 stimulation showed the functional MDSCs which induced IL-6 production, up-regulated ER stress sensor proteins by ER stress, enhanced the immune suppressive functions, and increased the ADAM17 expression. When the metastatic cancer cells expressed low level of GRP78, these cells lose the aggressive phenotype, invasion, and drug resistance, and decreased cancer growth *in vivo*.

Therefore, this study provides strong evidence that targeting GRP78 protein to inhibit the interaction of cancer cells and MDSCs in cancer microenvironment could be a novel approach to cancer therapy.

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Keywords: the 78 kDa glucose-related protein, breast cancer cell, myeloid-derived suppressor cells, metastasis, cancer microenvironment, ER stress, cancer-derived factor

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# INTRODUCTION

A cancer is an abnormal tissue in the body with distinct features. Through the development of specific growth conditions, cancers can promote the survival and tumorigenesis of cancer cells [1-4]. During tumorigenesis, the interaction of cancer and non-cancerous cells comprises the "cancer microenvironment" [42]. The non-cancerous cells involved in tumorigenesis include cancer-associated fibroblasts, myofibroblasts, endothelial cells, mesenchymal stem cells, and immune cells. These non-cancerous cells provide important support for cancer cell survival and growth. The cancer microenvironment plays a critical role in the modulation of cancer growth, metabolism, invasion, and metastasis to distant sites. Recent investigations of the cancer microenvironment also revealed the association of several immunosuppressive networks with anti-cancer immune responses. The formation of immunosuppressive networks results from the inhibition of anti-cancer immune cells, including T cells and antigen-presenting cells (APCs), such as dendritic cells (DCs) and monocytes/macrophages. Additionally, the reprogramming of myeloid cells creates an immunosuppressive phenotype [29]. The formation of immunosuppressive networks is promoted by soluble factors that are produced and released by cancer or non-cancerous cells within the cancer microenvironment [30]. Soluble factors can directly or indirectly influence cancer cells and subsets of immune cells that infiltrate the cancer microenvironment through a variety of mechanisms [31]. Recent reviews have focused on the role of the soluble factors present in the cancer microenvironment and their impact on immune cells. Cytokines and growth factors, such as vascular endothelial growth factor (VEGF), IL-6, and interleukin-10 (IL-10), are expressed by cancer cells, and these factors can inhibit dendritic cell (DC) differentiation and maturation [32-34].

Accumulation and secretion of oncometabolites caused by the cancer-specific metabolism within the cancer microenvironment impairs the DC function involved in the prevention of cancer cell immune invasion [35,36]. Several recent reviews focusing on the mechanisms leading to T cell dysfunction in solid cancers have been published. The opposing effects of IL-10 observed on T cell function might be concentration-dependent [37], and the immunosuppressive role of transforming growth factor beta (TGF-β) involves acting on cytotoxic T cells to inhibit the expression of cytotoxic gene products, namely, perforin, granzyme A and B, Fas ligand, and interferon (IFN)-$\gamma$ [38]. Chemokines, such as CXCL12 and CCL2, are relevant factors that prevent T cell infiltration [39,40] and support cancer cell immune escape. Cancer-derived soluble factors were shown to induce accumulation of the myeloid cells that aid cancer cells in migration and in the formation of new blood vessels in the cancer microenvironment [9−12]. Also, soluble factors promote the immune suppressive function with regard to myeloid cells including MDSCs [5-7].

Our previously elucidated a novel mechanism by which metastatic cancer cells induced higher infiltration of myeloid-derived suppressor cells [MDSCs] that influenced breast cancer cell aggressiveness and led to spontaneous metastasis. I found that cancer cells recruited MDSCs and prompted them to secrete interleukin-6 (IL-6). By comparing two different breast cancer cell lines (metastatic 4T1 and non-metastatic EMT6), I found that IL-6-expressing 4T1 cells showed extensive lung metastasis, whereas EMT6 cells showed no distant metastasis in the lung, liver, bone, or brain. 4T1 cells also showed dramatic recruitment of CD11b+Gr-1+ MDSCs in the spleen, liver, lung, and primary cancer mass. The critical role of IL-6 trans-signaling was confirmed because high IL-6 and soluble IL-6Ra secretion triggered a persistent increase of phospho-signal transducer and activator of transcription 3 (pSTAT3) in cancer cells [13,14]. However, when I further evaluated whether IL-6-mediated MDSC recruitment promoted the metastasis of

non-metastatic EMT6 breast cancer cells in mice, high IL-6 showed a minimal increase in distant lung metastasis. Therefore, I concluded that IL-6 secreted from breast cancer cells is sufficient for MDSC expansion and recruitment, but additional factors are required to facilitate the recruitment and metastasis of MDSC-mediated cancer cells. According to my study, metastatic murine 4T1 breast cancer cell expressed higher intracellular levels of the 78kDa glucose-regulated protein 78 (GRP78) than did EMT6 non-metastatic cancer cells. Accumulating evidence shows that cancer-derived factors and cancer-cell signaling mediators, such as Hsp72 and S1pr1, activate MDSCs to potentiate their immunosuppressive functions or increase the recruitment and colonization of these cells into pre-metastatic tissues [18,19]. Thus, I studied the characterization of GRP78 from 4T1 cell line, and the effect of GRP78 to accentuate 4T1 cancer progression via the modulation of MDSCs in the cancer microenvironment.

GRP78 (also known as binding immunoglobulin protein, BIP) is a glucose-regulated protein and is a member of the heat shock protein 70 family. GRP78 is a central chaperone located in the endoplasmic reticulum (ER) that protects cells under stress conditions, such as hypoxia, hypoglycemia, nutrient deprivation, infection, inflammation, and others [15]. In normal cells, GRP78 binds to PERK, IRE1, and ATF6 in the ER and maintains these proteins in an un-activated state. Under stressful conditions, GRP78 increases in the ER and binds unfolded or misfolded proteins. PERK, IRE1, and ATF6 are separated from GRP78 and induce the "unfolded protein response (UPR)" [24,25]. Despite its role as an ER retention chaperone with a KDEL motif, GRP78 is also present in the cell membrane, cytoplasm, mitochondria, nucleus, and extracellular environment. Cell surface GRP78 can bind the activated a2-macroglobulin (a2-M) and regulate the PAK2 activation mechanism for cancer survival and metastasis. It can also interact with Cripto, a GPI-anchored protein on the cell surface, and mediate the Cripto-dependent activation of c-Src, ERK, and Akt [16].

Up-regulation of GRP78 could lead to the translocation of GRP78 to cell surface membranes and therefore enhance its secretion to the extracellular environment, which has also been recognized to have extracellular functions [20–23]. The cellular translocation mechanism of GRP78 is less known, but GRP78 may be a prerequisite for its various locations. GRP78 is usually highly induced in poorly perfused cancers through the ER stress pathway caused by cancer-microenvironment factors, such as glucose deprivation, hypoxia, and acidosis. As an important cellular defense mechanism, high induced-GRP78 plays a role in apoptosis resistance, immune escape, drug resistance, metastasis, and angiogenesis of cancer cells [17–19]. Intracellular GRP78 promotes the activation of Akt in both cancer and leukemic cells [27,28]. GRP78 is a crucial link between cancer cells and the cancer microenvironment. Secreted GRP78 may stimulate the cancer microenvironment, including non-cancerous cells, and further facilitate cancer cell invasion, growth, and metastasis. GRP78 is also associated with tumorigenesis in cancer-associated macrophages (TAMs), termed M2 macrophages. M2 macrophages improve cancer progression and promote cancer metastases via the expression of GRP78 in cancer cells. Excessive GRP78 further induced the expression and secretion of interleukin 1 beta (IL-1β) and cancer necrosis factor alpha (TNFα) by activating STAT3, providing an inflammatory microenvironment for cancer growth and further promoting cancer cell migrations [12,23]. Moreover, soluble GRP78 can induce IL-10-producing splenic CD19+cells and IL-10 secretion as well as expression of PD-L1 and FasL as the important features of regulatory B cells [41].

I evaluated the characterization of intracellular GRP78 in cancer cells and extracellular GRP78 as soluble factors in the cancer microenvironment. According to my findings, metastatic 4T1 cancer cells expressed high levels of GRP78 for their survival and GRP78 was secreted into the cancer microenvironment. Secreted

GRP78 directly contributed to IL-6 production and ER stress induced by MDSCs. Upon treatment with GRP78, MDSCs increased the immune suppression. This resulted in high expression levels of PD-L1, high IL-10 secretion, and expression of ADAM17 in support of cancer cell aggressiveness and increased spontaneous metastasis. Intracellular GRP78 showed that a low level of GRP78 in metastatic cancer cells resulted in a loss of the aggressive phenotype and led to poor invasion, drug resistance, and decreased cancer growth *in vivo*. GRP78 accentuated metastatic cancer progression via the modulation of MDSCs in the cancer microenvironment to induce metastasis to distant site. Therefore, this study provides evidence that targeting GRP78 in cancer cells and inhibiting secretion of GRP78 within the cancer microenvironment could constitute a novel approach to cancer chemotherapy.

# MATERIALS AND METHODS

**Cell lines.**

The 4T1 murine breast cancer cell line (ATCC, CRL-2539) were purchased from the American Type Culture Collection (Manassas, USA) and cultured in RPMI 1640 (WELGENE, South Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, USA) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37℃ incubation with 5% $CO_2$. GRP78 low-expressing 4T1 #8 cells were established by limit dilution. Original 4T1 cells diluted in 96 well plates and selected #8 cells according to low IL-6 production from MDSC cultured with selected cell line conditioned medium. 4T1 #8 cells indicated low level of GRP78 expression and secretion.

**Conditioned medium (CM) preparation.**

Cells ($1\times10^4$) were incubated for 48 hours and the culture supernatants were collected.

**Animals.**

BALB/c mice were purchased from the Jackson Laboratory. Mice experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University (authorization no. SNU-161017-11).

**Cancer models.**

4T1 and 4T1 #8 cells ($2\times10^5$ /mouse) were injected into the mammary fat pads of BALB/c mice. Cancer growth was measured 2 times a week. To verify the effect of GRP78 of cancer growth, 4T1 #8 cells were orthotopically grafted into the mammary fat pad of BALB/c and GRP78 recombinant protein was administered continuously to cancer site using an Alzet osmotic mini-pump (0.2 mg/ml as 0.25 ul/ hr for 28 days).

**MDSC isolation.**

To isolate splenic MDSCs, mice were sacrificed 20-24 days after cancer cell injection and splenocytes were prepared from spleen of 4T1-bearing mice. MDSCs were purified with anti-CD11b and anti-Gr-1 mouse antibodies using a FACS Aria cell sorter (BD Biosciences). The sorting purity was greater than 95%.

**Flow cytometry.**

To analyze lymphocytes in cancer mass, cancer-bearing mice were sacrificed 20-24 days after cancer cell injection and cancers were prepared. Cancer tissue digested with 1 mg/ml of Collagenase D (Invitrogen, USA) and 0.5 U/ml of DNase I (Sigma-Aldrich, USA) for 1 hour at 37°C incubation. Cells were isolated by Percoll (Amersham, UK). Cells in 30% Percoll were added onto 70% Percoll and centrifuged for 20 minutes. The interface cells were retained and analyzed.

**Recombinant GRP78 production.**

Recombinant GRP78 protein was obtained from an Escherichia coli BL21 containing pET28a vector. Cloning product inserted into pET28a vector using enzyme BamHI and HindIII. These pET28a vector was inserted into BL21 and GRP78 protein production was promoted by ITPG induction. GRP78 in frame with the N-terminal 6×His-tag sequence purified using the His-tag purification Kit (Promega, USA). Endotoxins were removed by the Toxin Sensor$^{TM}$ Endotoxin Removal Kit (GenScript, USA), and the endotoxin level detected by LAL assay was less than 1 EU/mg. GRP78 proteins contained in 0.24% Tris, 7% Potassium chloride (KCl), 0.05% Magnesium chloride ($MgCl_2$), 0.015% DTT buffer (pH7.4). Concentrated GRP78 protein was detected by BCA Protein Assay and identified by SDS-PAGE.

**ELISA.**

MDSCs were cultured on a 96-well plate ($2\times10^5$ /well). Supernatants were collected and quantified cytokines. To detect soluble IL-6, anti-mouse IL-6 (eBioscience) as the capture antibody and biotinylated anti-mouse IL-6 (eBioscience) in 0.1% BSA in PBS/T as the detection antibody ed. Recombinant IL-6 protein (eBioscience) as the standard were used. To detect IL-10, anti-mouse IL-10 (R&D Systems) as the capture antibody, biotinylated anti-mouse IL-10 (R&D Systems) as the detection antibody and recombinant IL-10 protein (R&D Systems) as the standard were used. Absorbance was measured in a spectrophotometer at a wavelength of 570 nm.

**Western blotting.**

Cells were harvested in lysis solution and incubated on ice for 30 min. Cellular debris was removed by centrifugation. For soluble protein detection, prepared cell conditioned medium (CM) were concentrated using Amicon centricon centrifugal filter unit (Millipore, USA). Proteins were quantified by BCA protein assay. Proteins (10 μg) were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The membranes ware blocking with 5% skim milk for 1 hour and probed with an appropriate antibody; anti-β-actin (Sigma-Aldrich), anti-GRP78 (Cell Signaling). Develop was performed with chemiluminescence Western blotting detection system (Amersham, UK).

**RNA analysis.**

| Name | Primer sequence |
|---|---|
| GAPDH | F: 5'-GTCAGTGGTGG ACCTGACCT -3'<br>R: 5'-AGGGGTCTACATGGCAACTG-3' |
| GRP78 | F: 5'-AGT GGT GGC CAC TAA TGG AG-3'<br>R: 5'-CAA TCC TTG CTT GAT GCT GA-3' |
| GRP94 | F: 5'-CTG GGT CAA GCA GAA AGG AG-3'<br>R: 5'-TCT CTG TTG CTT CCC GAC TT-3' |
| Gadd34 | F: 5'-AGG ACC CCG AGA TTC CTC TA-3'<br>R: 5'-AGG TAG GGA CCC AGC TTC TC-3' |
| CHOP | F: 5'-CAT ACA CCA CCA CAC CTG AAA G-3'<br>R: 5'-CCG TTT CCT AGT TCT TCC TTG C-3' |
| XBP-1 | F: 5'-AGT TAA GAA CAC GCT TGG GAA T-3'<br>R: 5'-AAG ATG TTC TGG GGA GGT GAC-3' |
| IL-6 | F: 5'-GACAAAGCCAGAGTCCTTCAGAG-3'<br>R: 5'-CTAGGTTTGCCGAGTAGATCTC-3' |
| IL-23p19 | F: 5'-CAG CAG CTC TCT CGG AAT CT-3'<br>R: 5'-CTG GAG GAG TTG GCT GAG TC-3' |
| PD-L1 | F: 5'-GAG TGC AGA TTC CCT GTA GAA C-3'<br>R: 5'-ACC CTC GGC CTG ACA TAT TA-3' |
| Adam17 | F: 5'-GAG GAG TGT GAC CCG GGT A-3'<br>R: 5'-GGG GGC ACT CAC TGC TAT T -3'. |

The total RNA was isolated from MDSCs using the RNeasy kit (QIAGEN; 74104). cDNA was synthesized from 1 μg of total RNA by M-MLV reverse transcriptase (TAKARA, Japan). PCR reactions were performed with the following primer pairs and then PCR product were analyzed by 1.5% agarose gel electrophoresis.

**T Cell Suppression Assay.**

CD8+ T cells were isolated from the spleen of BALB/c mice and purified using MACS. T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Life Technologies, Belgium). Splenic MDSCs were obtained from splenocytes of 4T1-bearing mice with anti-CD11b and anti-Gr-1 by MACS.

In a U-bottom 96-well cell culture plate with 2 ul of anti-CD3 /anti-CD28 Dyanead beads, $2\times10^5$ purified T cells were cultured in DMEM medium with 10% FBS for 96 h. Isolated splenic MDSC were added to the stimulated T cells according to the indicated ratios with recombinant GRP78. Dilution of CFSE was evaluated 3 days later by flow cytometry as the measure of T cell proliferation. Data were collected using the FACS LSRII Forttesa (Becton Dickinson) and were analyzed with FlowJo10 (Treestar Inc.).

**Invasion Assay.**

Cells ($5\times10^4$) with Matrigel matrix (250 μg/ml, Matrigel™ Basement Membrane Matrix, BD Bioscience) were seeded into the upper chamber of the Transwell (FALCON) and then the lower chamber was filled with collagen matrix (5 μg/ml). Invasion assays were carried out for 48 h. Non-invading cells on top of the matrix

were removed by rubbing with a moistened cotton swab. Invaded cells on the lower surface of the Matrigel matrix were fixed with 4% PFA and stained with 0.2% crystal violet and counted using ImageJ software (version 1.46)

**Suspension culture.**

Suspension culture of cells ($5\times10^3$/well) was carried out for 4 days. Cell culture plates were coated with 12 mg/ml concentration of Poly-HEMA for 24 hours at 37°C incubation.

**MTT assay.**

Cell viability was measured by the MTT assay. Cells ($5\times10^4$) were seeded for 24 hour, and culture medium with different concentrations of the drugs (doxorubicin, mitoxantrone, paclitaxel and cisplatin) was replaced. After 48 or 72 hour incubation, cells were washed twice with PBS and incubated with 0.5 mg/ml MTT working solution at 37°C for 4 hour. Dimethyl sulfoxide (DMSO) was added for 10 min and absorbance was measured in a spectrophotometer at a wavelength of 570 nm. Data was expressed as means ± SEM of each group. The p values were obtained using unpaired two-tailed Student′s.

**Statistical analyses.**

Statistical significance was measured by one-way analysis of variance (ANOVA).

Data was analyzed using the GraphPad prism software (version 5.0). P value was considered as significance and standard errors was presented in SEM.

# Results

**Selection of GRP78 as the main factor in a metastatic 4T1-derived molecule**

The 4T1 and EMT6 murine cell lines were investigated using a syngeneic (BALB/c) mouse xenograft model. The murine metastatic breast cancer 4T1 cell line had a high accumulation of myeloid cells in the spleen, lymph nodes, and cancer sites. In contrast, the murine non-metastatic breast cancer EMT6 cell line had few myeloid cells.

Our previous report revealed that the recruitment of MDSCs by metastatic 4T1-mediated IL-6 production from the bone marrow to the cancer environment facilitated the invasion and migration of cancer cells [14]. To investigate additional factors that recruit and facilitate MDSC-mediated metastasis, I examined other 4T1-derived factors.

4T1 cells were incubated for 48 hours, and 4T1-derived proteins were harvested from 4T1 conditioned media (CM). Splenic MDSCs were isolated from 4T1-bearing BALB/c mice. IL-6 production from MDSCs was regarded as a "read-out" of MDSC activation as defined by our previous work [14]. Splenic MDSCs were immature and inactivated without the contact with cancer cells in the cancer microenvironment. To search the major candidates, proteins from 4T1 CM were separated according to whether they were over or under 50kDa in size and cultured with splenic MDSCs. 4T1-CM proteins that were over 50kDa in size were validated in their ability to induce IL-6 production from splenic MDSCs (Fig. 1A). Additionally, the exosome forms of the 4T1-CM proteins did not induce IL-6

production from splenic MDSCs in comparison to the whole CM and supernatant fractions (Fig. 1B).

The proteomic data from the supernatant proteins of 4T1-CM and EMT6 cell lines were also analyzed to reveal major common proteins (Fig. 1C). Among a total of 715 proteins, 72 from 4T1-CM and 192 from EMT6-CM were upregulated. To identify proteins that were expressed at higher levels in the metastatic cancer microenvironment, 11 major proteins were selected based on the following three criteria: 1) the proteins were upregulated only in the 4T1-CM supernatant and not the EMT6-CM, 2) there was at least a three-fold change compared to the control, and 3) the protein was over 50 kDa in size (Fig. 1D). From these proteins, the 78kDa glucose-regulated protein (GRP78) was selected as the major factor to comprise and activate MDSCs and induce IL-6 production of MDSCs for the metastasis of cancer cells.

**4T1-derived GRP78 plays a critical role in MDSC-induced IL-6**

To reconstitute the role of GRP78 in 4T1-CM and IL-6 production from MDSCs, a GRP78-knockdown 4T1 cell line was established by transfecting 4T1 cells with siGRP78. The IL-6 production from siGRP78-4T1-CM-treated splenic MDSCs was lower compared to that from scrambled 4T1-CM-treated splenic MDSCs (Fig. 2A). To confirm the soluble GRP78 effect, a recombinant GRP78 protein was produced from *Escherichia coli (E. coli)*. After the incubation of splenic MDSCs for 24 hours with increasing concentrations of recombinant GRP78 protein, IL-6 production from splenic MDSCs increased in a dose-dependent manner (Fig 2B). To confirm the role of GRP78 in IL-6 production, splenic MDSCs were cultured with 4T1-CM (50% of total culture medium) and a GRP78-blocking reagent relative to the concentration. The blocking reagent decreased IL-6

production from GRP78-treated splenic MDSCs in a dose-dependent manner (Fig. 2C). Thus, soluble GRP78 plays a main role in MDSC IL-6 production within the cancer microenvironment.

**Functions of GRP78-treated splenic MDSCs**

To verify the functional differentiation of MDSCs with GRP78, I analyzed the microarray data of splenic MDSCs groups and cancer MDSCs. Splenic MDSCs from 4T1-bearing spleen (sMDSC) and splenic MDSCs were cultured with recombinant GRP78 protein (5 μg/ml, rGRP78) and 4T1-CM (50% of total culture medium, 4T1-CM) for 24 hours. Cancer MDSCs (tMDSC) were harvested from the 4T1 primary cancer mass. According to the PCA analysis, GRP78-treated splenic MDSCs was similar to 4T1-CM-treated splenic MDSCs (Fig. 3A) that GRP78 may be the main factor in the 4T1-CM as a 4T1-derived factor. Additionally, the heat map revealed the up-regulated genes as $\log_2$(Expression vs. sMDSC)>1.5(fold change) and the down-regulated genes as $\log_2$(Expression vs. sMDSC)>1.5(fold change) (Fig. 3B). Gene ontology description through ClueGO functional annotation chart showed that 4T1-CM and rGRP78 splenic MDSCs had a lot of functional properties; Positive regulation of interleukin-6 production, positive regulation of cytokine secretion, positive regulation of peptide secretion, chronic inflammatory response, negative regulation of extrinsic apoptotic signaling pathway, positive regulation of cell-cell adhesion, negative regulation of response to external stimulus, cellular response to TNF, cellular response to IL-1, macrophage migration, I-kappaB kinase/NF-kappaB signaling, activation of innate immune response, and pattern recognition receptor signaling pathway, et al (Fig. 3C).

**GRP78 stimulation induced ER stress on splenic MDSCs**

Recent studies have suggested that myeloid cells and cancer cells undergo ER stress by cancer cell-derived GRP78 during cancer growth [24,25]. GRP78-treated MDSCs showed the XBP1 gene ontology description through ClueGO functional annotation chart (Fig. 4A), and the functions related to ER stress, the regulation of programmed apoptosis and cell death, the defense response, and the unfolded protein response (UPR) (Fig. 3C).

To confirm whether soluble GRP78 induced ER stress on MDSCs, splenic MDSCs were co-cultured with different concentrations of recombinant GRP78 for 24 hours. The gene expression of the main ER stress sensor protein CHOP increased with increasing recombinant GRP78 in a dose-dependent manner (Fig. 4A). When thapsigargin (THP) was used as an ER stress inducer, the gene levels of other ER stress sensor proteins, such as GRP78, Gadd34, IL-6, and IL-23p19, were also up-regulated in GRP78-treated splenic MDSCs, but GRP94 was not different each other (Fig. 3C). These results show that soluble GRP78 induced ER stress in MDSCs and that the appropriate response facilitates the survival of MDSCs in the cancer microenvironment.

**GRP78 enhanced the immune suppression effect on MDSCs**

Based on the immune response function of GRP78-treated splenic MDSCs (Fig. 3C), there were 2 up-regulated genes, the PD-L1 immune checkpoint molecule that induced PD-1+-exhausted T cells and the HAVCR2 (TIM-3), showed the group functional chart (Fig 5A). The PD-L1 had 10 times higher gene expression in GRP78-treated splenic MDSCs compared to untreated (NT) splenic MDSCs (Fig. 5B). There was no difference in the fold change of PD-L2 between GRP78-treated and untreated splenic MDSCs. Moreover, PD-L1 genes from

GRP78-treated splenic MDSCs showed 30 times higher expression in GRP78-treated splenic MDSCs and 40 times higher expression in cancer MDSCs compared to untreated splenic MDSCs (Fig. 5B).

To evaluate the immune suppressive function of GRP78-treated splenic MDSCs, T cells and MDSCs were co-cultured. Syngeneic T cells and splenic MDSCs were incubated with or without recombinant GRP78, using anti-CD3/anti-CD28 beads for T cell stimulation. T cell proliferation was measured through CFSE labeling. Treatment with recombinant GRP78 protein did not change T cell proliferation without MDSC co-culture (0:1). However, MDSCs could sufficiently suppress T cell proliferation, even with anti-CD3/anti-CD28 T cell stimulation, and CD8+ T cell proliferation was more suppressed when incubated with GRP78 (Fig. 5C). GRP78 had a direct effect on MDSCs and an indirect effect on T cells. Additionally, recombinant GRP78 protein increased IL-10 production of splenic MDSCs in a dose-dependent manner (Fig. 5D). These data suggest that soluble GRP78 activates splenic MDSCs and that this enhances the immune suppressive response in support of cancer cell survival in the cancer microenvironment.

**GRP78-treated MDSCs support the migration and metastasis of cancer cells**

Because GRP78-treated splenic MDSCs showed an increased response to wound function (Fig. 3C), the gene expression of ADAM17 from the Adam-family proteases was showed the group functional chart (Fig. 6A). Microarray analysis showed that ADAM17 gene expression was higher in GRP78-treated splenic MDSCs compared to untreated (NT) splenic MDSCs (Fig. 6B). Also, ADAM17 gene expression from GRP78-treated splenic MDSCs, measured by RT-PCR, showed four-fold higher expression compared to untreated splenic MDSCs (Fig. 6B). The expression of ADAM17 on GRP78-treated splenic MDCSs facilitates not

only the wounding of cancer cells but also the release of the IL-6 receptor. Therefore, it contributes to the invasive nature of cancer cells and distant metastasis through IL-6 trans-signaling.

**Comparison of 4T1 #8 cells with low GRP78 expression with original 4T1 cells**

The extracellular function of cancer-derived GRP78 as the soluble factor to activate MDSCs in cancer microenvironment was also evaluated. A low-expressing GRP78 4T1 cell line was generated from several limited dilutions of original 4T1 cells (4T1-Ori). Original 4T1 cells were diluted in 96-well plates, and cell lines were selected according to IL-6 production from MDSCs cultured with the selected cell line conditioned medium (CM). 4T1 #8-CM induced low IL-6 production from MDSCs compared to 4T1-Ori-CM (Fig. 7A). Furthermore, selected 4T1 #8 cells had low overall expression levels of intracellular GRP78 protein, and the 4T1 #8-CM group had low levels of secreted GRP78 protein (Fig. 7B). Surface and intracellular GRP78 from 4T1 #8 cells were detected by flow cytometry (Fig. 7C). The gene level of the heat shock protein and chaperone GRP94 in 4T1 #8 cells was similar to that in 4T1-Ori cells (Fig. 7D). Importantly, the characterization of cells that express low levels of GRP78 (4T1 #8 cells) helps to evaluate cancer-derived intracellular and extracellular GRP78 in the cancer microenvironment.

**4T1 #8 cells *in vitro* have fewer cancer stem cell-like characteristics**

Recent research has demonstrated the critical role of GRP78 in cancer cell proliferation, survival, and cancer angiogenesis [17,18]. The 4T1 #8 cell line had low intracellular GRP78 in the ER, nucleus, mitochondria, and cytoplasm. Moreover, the GRP78 extracellular soluble protein was also low in the cell line.

Thus, when the cancer cells had low intracellular GRP78, they could not secret GRP78 into the cancer microenvironment.

To confirm the function of intracellular GRP78 in cancer cells, the differential phenotypes of 4T1-Ori and 4T1 #8 cell lines were investigated (Fig. 8A). The 4T1-Ori cell line had a morphology that was long and sharp, but these aggressive phenotypes were diminished in the 4T1 #8 cells. To evaluate the biological behavior and migration of the cells, a matrigel invasion assay was performed. 4T1 #8 cells showed decreased invasiveness compared to 4T1-Ori cells (Fig. 8B). Suspension cultures of 4T1- Ori and 4T1 #8 cells on Poly-HEMA-coated plates were also carried out for 4 days. 4T1-Ori cells formed spheres, whereas 4T1 #8 cells showed cell aggregation without sphere formation (Fig. 8C). These findings showed that 4T1 #8 cells were insufficient for cancer formation and the components needed to establish the cancer microenvironment because of the low intracellular GRP78.

Next, to address the effects of intracellular GRP78 on the characteristics of cancer stem cells that lead to drug-resistance, 4T1-Ori and 4T1 #8 cells were treated with different concentrations of cancer drugs (doxorubicin, mitoxantrone, paclitaxel, or cisplatin). The cell viability of 4T1-Ori and 4T1 #8 cells was measured using the MTT assay after 48–72 hours of incubation. All four drugs resulted in lower 4T1 #8 cell viability compared to 4T1-Ori cells (Fig. 8D). However, the cell viability after 5-fluorouracil treatment did not show a difference in resistance between 4T1-Ori and 4T1 #8 cells. These data revealed that cells with down-regulated GRP78 do not adopt drug resistance in 4T1 cancer cells and could be a new target for cancer chemotherapy within the cancer microenvironment.

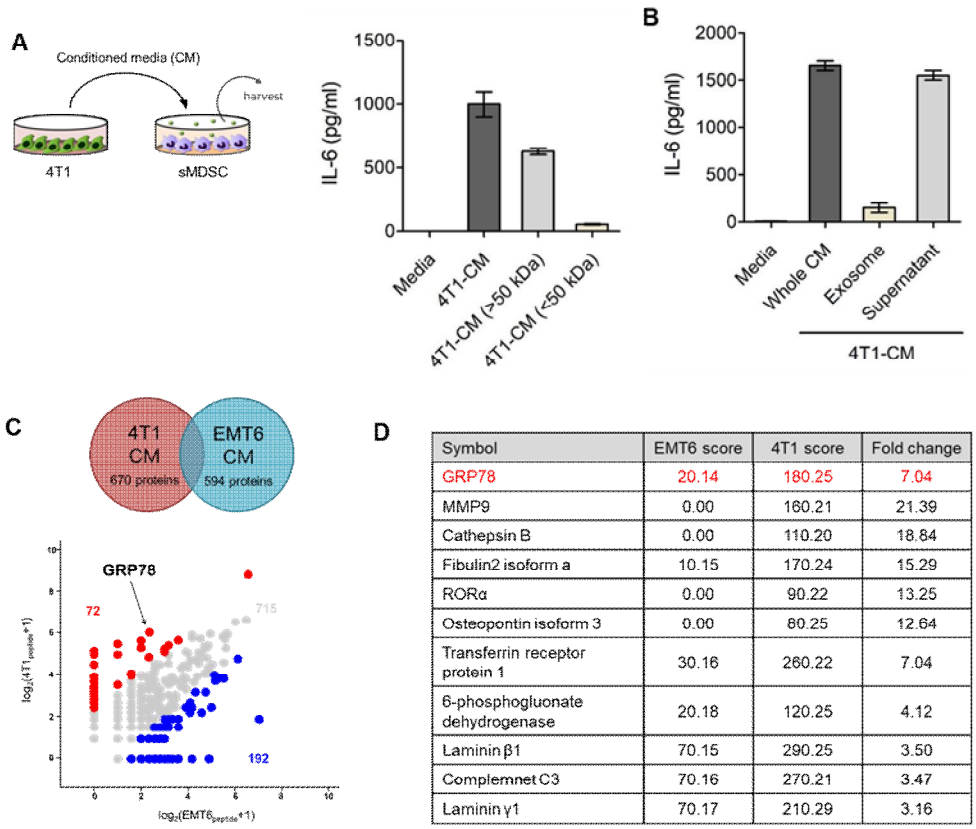
**4T1 #8 cell have reduced cancer growth *in vivo***

To verify the effect of GRP78 on cancer growth, 4T1-Ori and 4T1 #8 cells were injected into the mammary fat pads of BALB/c mice (Fig. 9A). Primary cancer growth was dramatically reduced in 4T1 #8 cells compared to 4T1-Ori cells during the experimental period. 4T1 #8 cells also showed no distant metastasis in the lung, liver, and spleen, whereas 4T1-Ori cells showed lung metastasis (Fig. 9B). The 4T1 #8-bearing mice had much smaller cancer sizes than did the 4T1-Ori-breaing cancer mice. Additionally, the 4T1 #8-bearing mice had much a smaller spleen size than did the 4T1-Ori-breaing mice due to fewer recruited MDSCs. The 4T1 #8-bearing mice had an extremely low population of CD11b+Gr-1+ MDSCs on the primary cancer mass; the total number of MDSCs was 10 times lower in 4T1 #8-bearing mice than in 4T1-Ori-bearing mice. The CD4+ and CD8+ T cell populations were larger in 4T1 #8 primary cancer-bearing mice, but they did not significantly differ from those in the 4T1-Ori primary cancer (Fig 9C–D). Thus, cancer-derived GRP78 is associated with MDSCs and facilitates cancer growth and metastasis.

**Restoration of cancer growth by induction of GRP78 *in vivo***

Next, the re-introduction of GRP78 in the 4T1 #8 cancer environment and its effect on recovery, cancer growth, and metastasis were evaluated. 4T1 #8 cells were orthotopically grafted into the mammary fat pad of BALB/c mice. GRP78 was continuously allowed into the cancer microenvironment using an Alzet osmotic pump that released recombinant GRP78 protein (0.2 mg/ml as 0.25 μl/ hr) to the primary cancer site of 4T1 #8-bearing mice starting from the day following cancer cell injection. Primary cancer growth was increased after adding GRP78 to 4T1 #8 cells compared to control 4T1 #8 cells during the experimental period (Fig. 10A). Adding GRP78 to 4T1 #8-bearing mice also increased cancer and spleen size

(Fig. 10B) and lung metastasis (Fig. 10C) compared to the control 4T1 #8 mice. Adding GRP78 to the 4T1 #8 cancer mass made less of a difference in the population of CD11b+Gr-1+ MDSCs compared to 4T1-Ori and 4T1 #8 cancer mass. However, there was a difference in the T cell population depending on the cancer size and the absolute number of cancers (Fig. 10D). The total cell and MDSC number on the cancer mass was two times higher in 4T1 #8-bearing mice given GRP78 than in 4T1 #8-bearing mice (Fig. 10E). Compared to the 4T1-Ori cancer, CD44 was upregulated on CD4 and CD8 T cells from the 4T1 #8 cancer. CD44 on CD4 and CD8 T cells from the 4T1 #8 cancer with GRP78 were down-regulated compared to those from the 4T1 #8 cancer following T cell activation (Fig. 10F). These findings indicate that adding GRP78 as a soluble factor on a cancer may restore cancer growth and metastasis due to the induction of ER stress and T cell suppression by activated MDSCs related to MDSC-mediated metastatic cancer growth.




| Symbol | EMT6 score | 4T1 score | Fold change |
|---|---|---|---|
| GRP78 | 20.14 | 180.25 | 7.04 |
| MMP9 | 0.00 | 160.21 | 21.39 |
| Cathepsin B | 0.00 | 110.20 | 18.84 |
| Fibulin2 isoform a | 10.15 | 170.24 | 15.29 |
| RORα | 0.00 | 90.22 | 13.25 |
| Osteopontin isoform 3 | 0.00 | 80.25 | 12.64 |
| Transferrin receptor protein 1 | 30.16 | 260.22 | 7.04 |
| 6-phosphogluonate dehydrogenase | 20.18 | 120.25 | 4.12 |
| Laminin β1 | 70.15 | 290.25 | 3.50 |
| Complemnet C3 | 70.16 | 270.21 | 3.47 |
| Laminin γ1 | 70.17 | 210.29 | 3.16 |

**Figure 1. Selection of GRP78 as the main factor in a metastatic 4T1-derived molecule**

(A) 4T1 cells were incubated for 48 hours with serum-free conditioned media, and the culture supernatant was collected. Proteins in 4T1-CM were divided into two groups (>50kDa size and <50kDa size) using the Amicon Centricon centrifugal filter unit. The divided 4T1-CM was used to treat splenic MDSCs. 4T1-CM-MDSCs were cultured for 24 hours, supernatants were collected, and IL-6 was quantified ELISA.

(B) Proteins in 4T1-CM were divided into an exosome protein form, supernatant, and whole proteins. These divided 4T1-CM groups were used to treat splenic MDSCs. 4T1-CM-MDSCs were cultured for 24 hours, and supernatants were collected for IL-6 quantification by ELISA.

(C) Proteomic data about the proteins expressed in 4T1-CM and EMT6-CM cells. 4T1 and EMT6 cells were incubated for 48 hours with serum-free conditioned media, and the culture supernatant was collected.

(D) Top 11 proteins were chosen based on high up-regulation by 4T1-CM but not EMT6-CM, greater than 3.0-fold change, over 50kDa in size, and the non-exosome form of the protein.

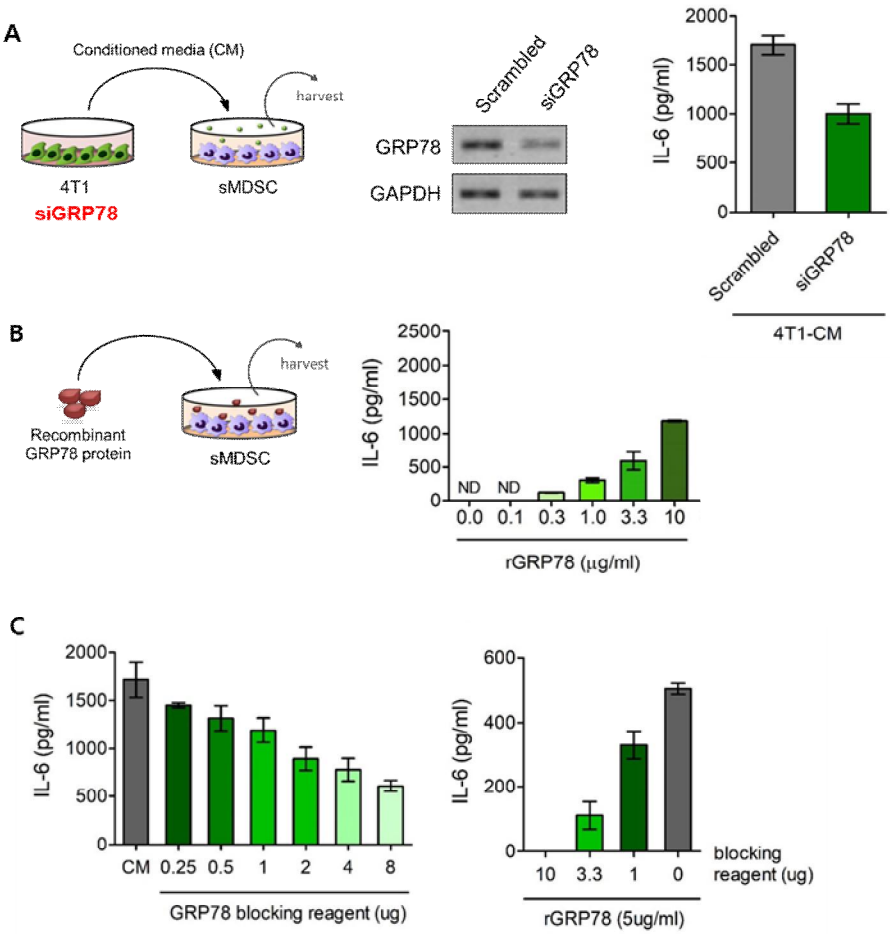



**Figure 2. GRP78 plays a critical role in MDSCs-induced IL-6**

(A) A siGRP78 vector was used for the transient transfection of 4T1 cells. GRP78 expression of siGRP78-transfected 4T1 cells was measured by Western blotting. Scrambled or siGRP78-treated 4T1 cells were cultured for 48 hours and then harvested in conditioned medium (50% of total medium volume) and used to treat splenic MDSCs. IL-6 levels in the culture supernatant of splenic MDSCs co-cultured with scrambled or siGRP78 4T1-CM were measured by ELISA.

(B) IL-6 levels in the culture supernatant of splenic MDSCs. Splenic MDSCs were incubated for 24 hours with the different concentrations of recombinant GRP78 protein, and IL-6 levels were measured by ELISA.

(C) IL-6 levels of splenic MDSCs incubated for 24 hours with different concentrations of GRP78 blocking reagent (left Fig), plus recombinant GRP78 protein (right Fig). IL-6 levels from the culture supernatant of splenic MDSCs were measured by ELISA.

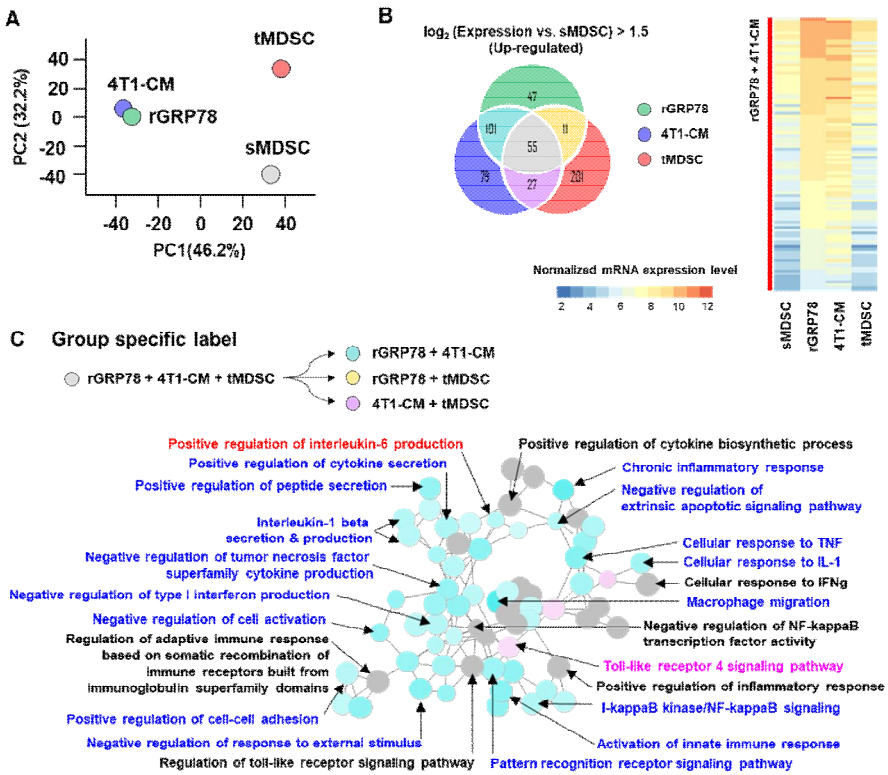



**Figure 3. Functions of GRP78-treated splenic MDSCs**

(A) PCA analysis; PC1 46.2%, PC32.2%
Splenic MDSCs from 4T1-bearing spleen (sMDSC) and splenic MDSCs were cultured with recombinant GRP78 protein (5 μg/ml, rGRP78) and 4T1-CM (50% of total culture medium, 4T1-CM) for 24 hours. Cancer MDSCs (tMDSC) were harvested from the 4T1 primary cancer mass.

(B) Heat maps; $log_2$(Expression vs. sMDSC)>1.5(fold change) (Up-regulated), $log_2$(Expression vs. sMDSC)<1.5(fold change) (Down-regulated), and normalized mRNA expression level.

(C) ClueGO functional annotation chart through gene ontology description

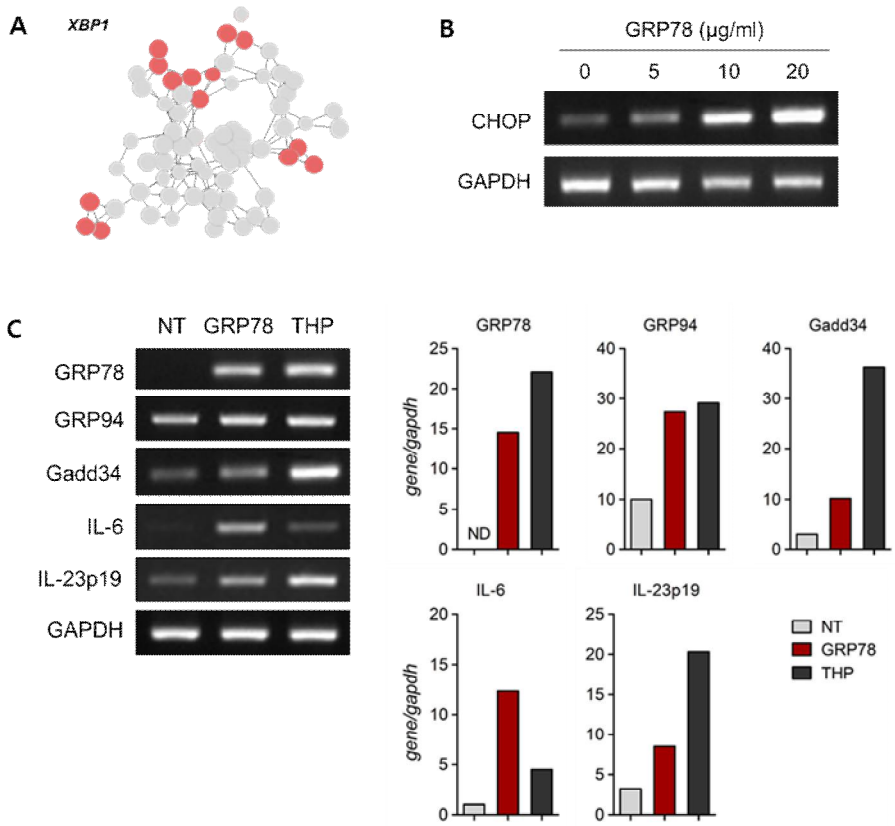



**Figure 4. GRP78 stimulation induced ER stress on splenic MDSCs**

(A) XBP1 gene ontology description through the ClueGO functional annotation chart

(B) The transcription levels of CHOP and GAPDH genes in GRP78-treated splenic MDSCs were measured by RT-PCR. Splenic MDSCs were co-cultured with recombinant GRP78 with increasing concentrations for 24 hours and harvested.

(C) The transcriptional levels of genes in splenic MDSCs were measured by RT-PCR. Splenic MDSCs were co-cultured with recombinant GRP78 (5 μg/ml, 10 μg/ml) and Thapsigargin (THP, 10 nM) for 24 hours and harvested.

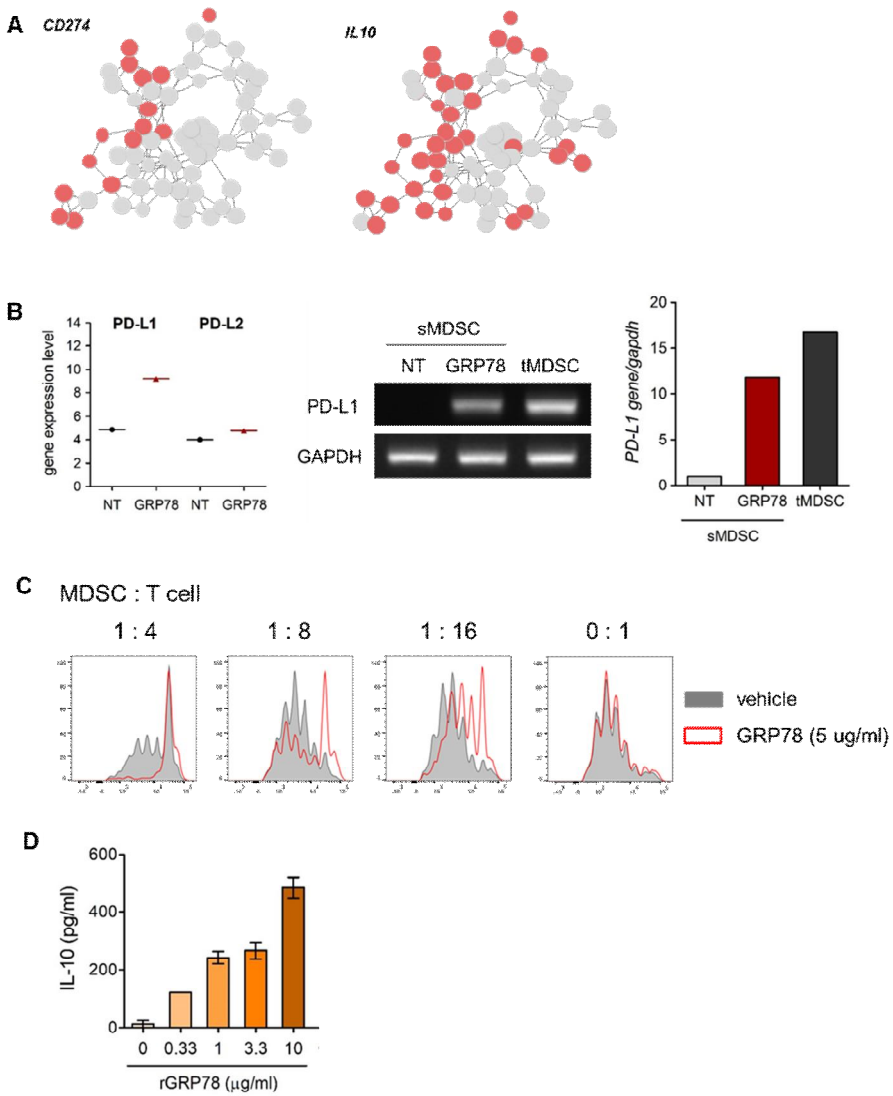



**Figure 5. GRP78 enhanced the immune suppression effect on MDSCs**

(A) CD274 and HAVCR2 gene ontology description through the ClueGO functional annotation chart

(B) The fold change levels of PD-L1 and PD-L2 genes in GRP78-treated splenic MDSCs compared to those of untreated splenic MDSCs (NT). The transcription levels of the PD-L1 gene in GRP78-treated splenic MDSCs were compared to those of untreated splenic MDSCs (NT) by RT-PCR. Splenic MDSCs were co-cultured with recombinant GRP78 (5 μg/ml) for 24 hours, and cancer-MDSCs were harvested from the 4T1 primary cancer. The values of PD-L1 were normalized to the GAPDH loading control.

(C) CFSE-labeled T cells were co-cultured with splenic MDSCs in a U-bottom 96-well plate with 2 μl of anti-CD3/anti-CD28 Dyanead beads for T cell stimulation.

Dilution of CFSE was evaluated 3 days later by flow cytometry as a measure of T cell proliferation. The gray color of graphs represents T cell stimulation only, and the red lines represent cells with the addition of recombinant GRP78 (5 μg/ml).

(D) IL-10 levels in a culture supernatant of splenic MDSCs treated with recombinant GRP78 with increasing concentrations measured by ELISA.

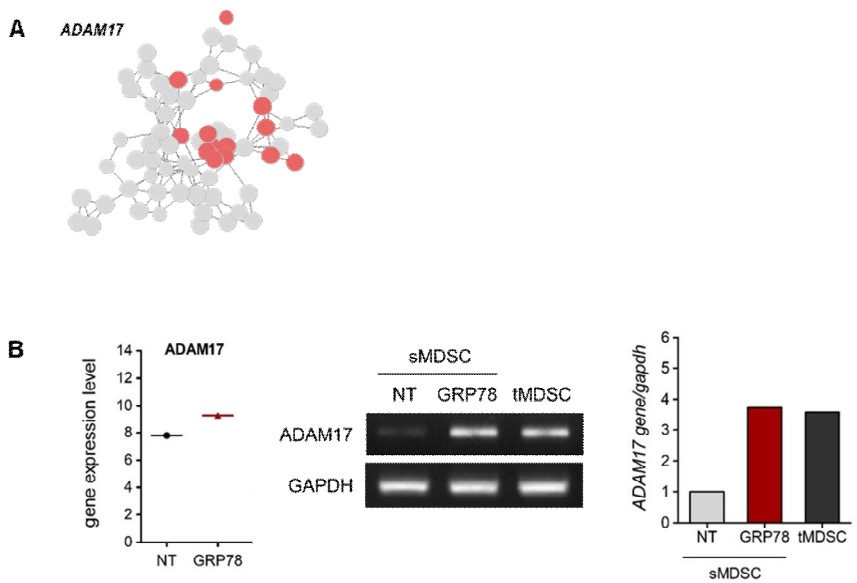



**Figure 6. GRP78-treated MDSCs support migration of cancer cells**

(A) ADAM17 gene ontology description through the ClueGO functional annotation chart

(B) The fold change levels of the upregulated ADAM17 gene in GRP78-treated splenic MDSCs compared to those of untreated splenic MDSCs (NT). The transcription levels of the ADAM17 gene in GRP78-treated splenic MDSCs were compared to those of untreated splenic MDSCs (NT) by RT-PCR. Splenic MDSCs were co-cultured with recombinant GRP78 (5 μg/ml) for 24 hours, and cancer-MDSCs were harvested from the 4T1 primary cancer. The values of PD-L1 were normalized to the GAPDH leading control.

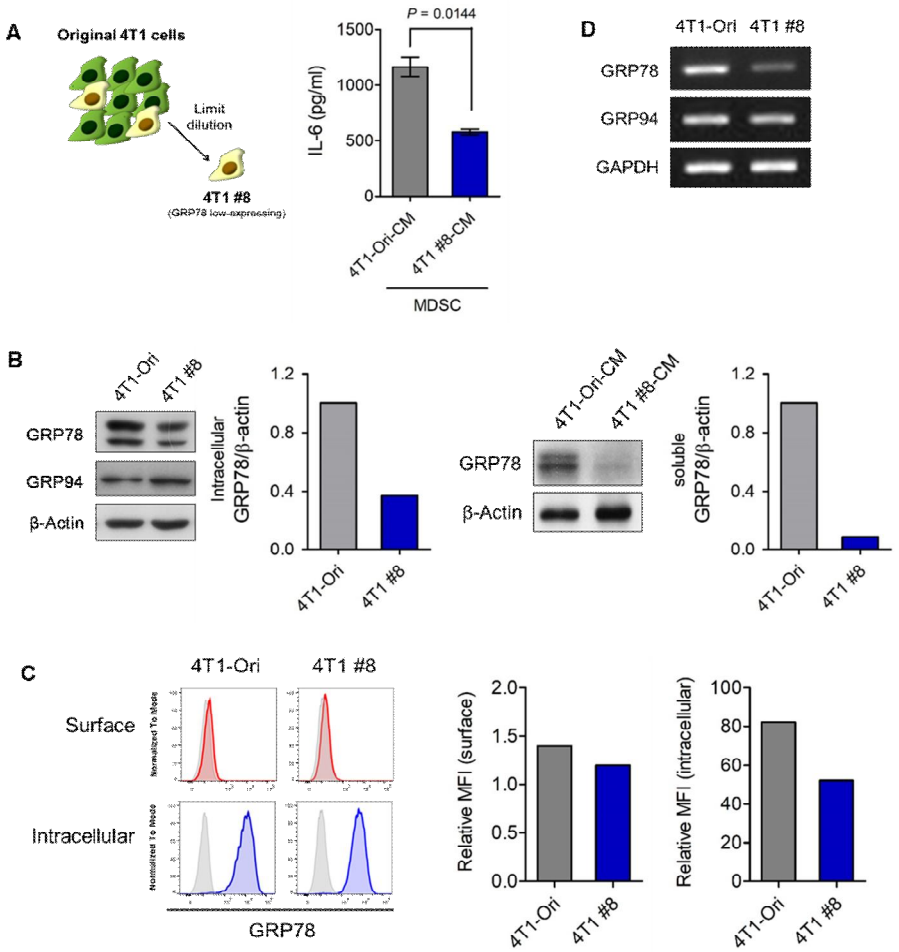



**Figure 7. Comparison of 4T1 #8 cells with low GRP78 expression with original 4T1 cells**

(A) Original 4T1 cells diluted to 0.3 cells/well in 96-well plates and selected #8 cells according to low IL-6 production by splenic MDSCs cultured with selected 4T1 #8-CM.

(B) The overall protein levels of GRP78 were measured by Western blotting. The culture supernatant was harvested, and the secreted levels of GRP78 were detected by Western blotting.

(C) The surface and intracellular expression levels of GRP78 on 4T1-Ori and 4T1 #8 cells were determined by flow cytometry. The graph represents the relative mean fluorescence intensity (MFI) of intracellular GRP78 expression. *Relative MFI = sample MFI/FMO control MFI

(D) The transcriptional levels of GRP78, GRP94, and GAPDH on 4T1-Ori and 4T1 #8 cells measured by quantitative RT-PCR.

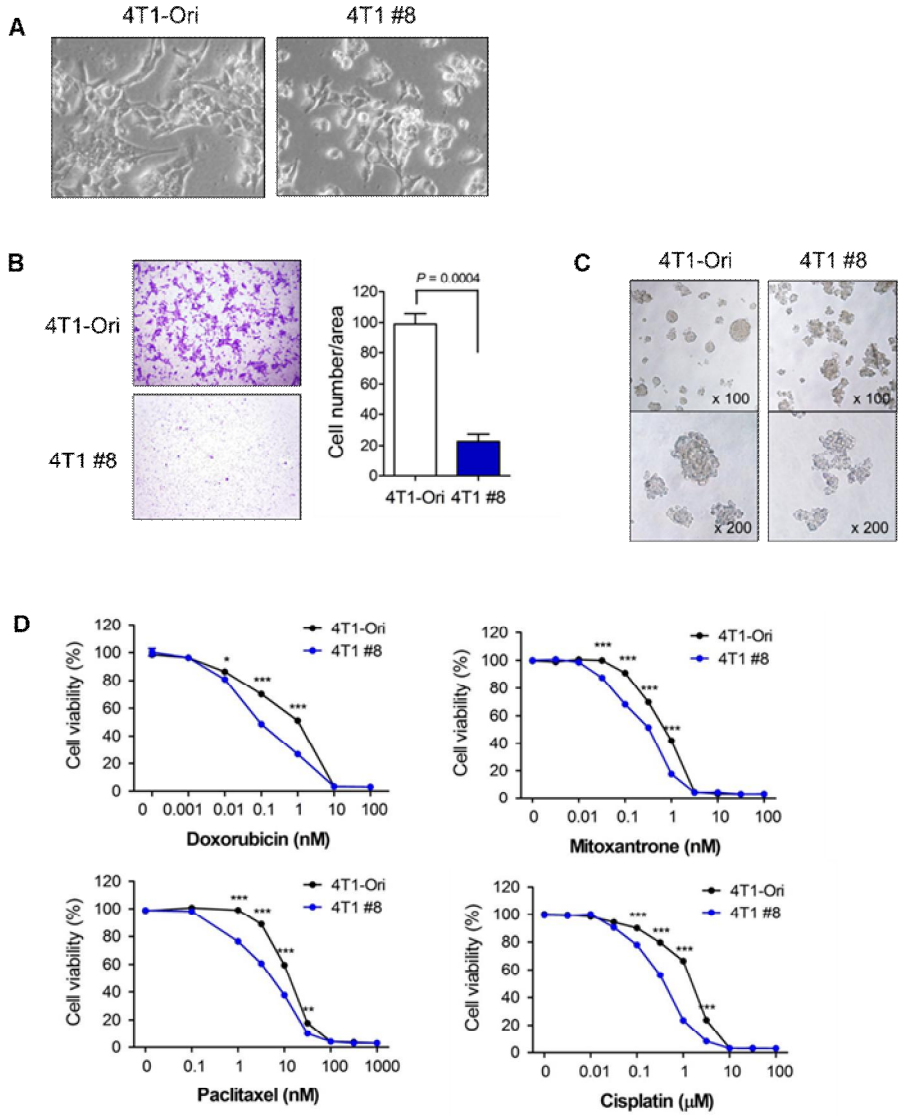



**Figure 8. 4T1 #8 cells *in vitro* have fewer cancer stem cell-like characteristics**

(A) Representative microscopic images of Original 4T1 cells (4T1-Ori) and GRP78 low-expressing 4T1 #8 cells (4T1 #8).

(B) Cell invasion assays. Matrigel matrix was used at a 250 μg/ml concentration with cells placed into the upper chamber of the Transwell. Non-invading cells on top of the matrix were removed, and invaded cells on the lower surface of the Matrigel matrix were fixed, stained, and counted 48 hours later.

(C) Suspension culture images of 4TI-Ori and 4T1 #8 cells. Cell culture plates were coated with a 12-mg/ml concentration of Poly-HEMA. Suspension culture of 4T1-

Ori and 4T1 #8 cells was carried out for 4 days.

(D) Cell viability of 4T1-Ori and 4T1 #8 cells treated with the different concentrations of drugs: (A) doxorubicin, (B) mitoxantrone, (C) paclitaxel, or (D) cisplatin; concentrations were measured by MTT assay. The p values were obtained using two-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

All data shown are representative of three independent experiments using samples from triplicate cell cultures.

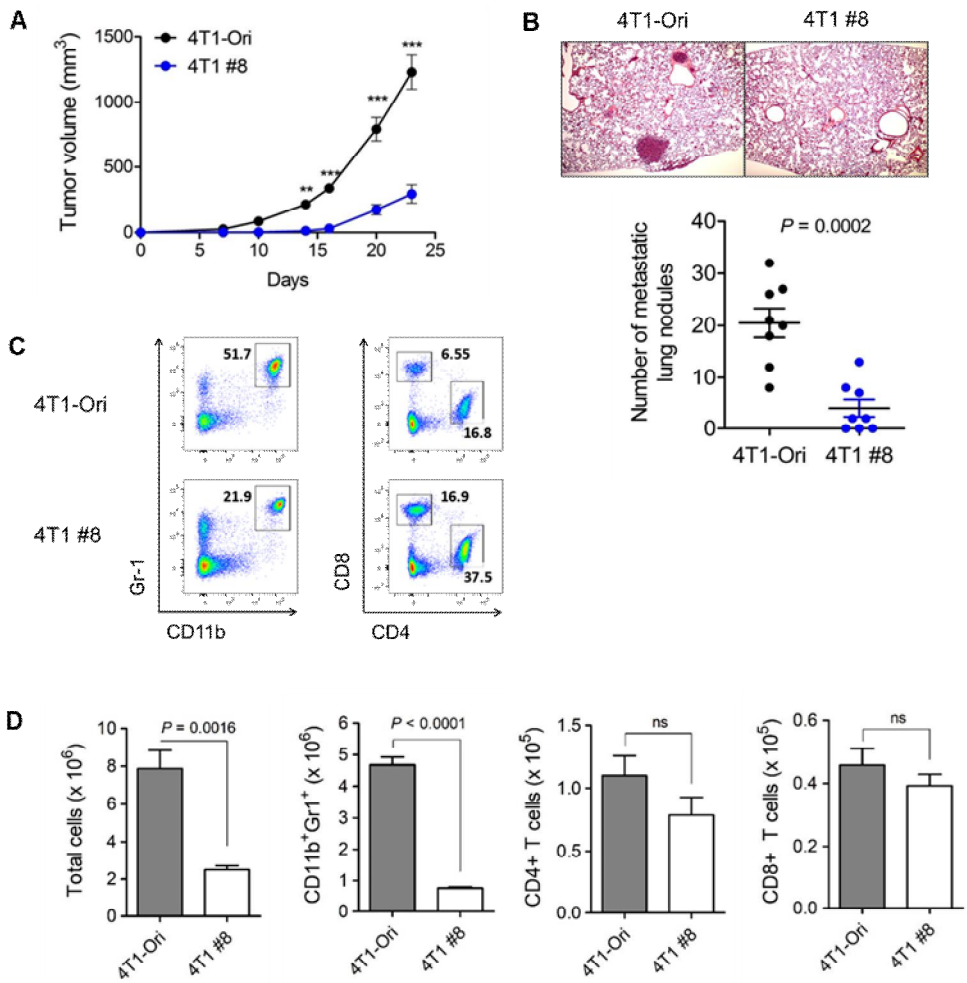



**Figure 9. Reduced cancer growth in 4T1 #8 cancer *in vivo***

*In vivo* cancer growth of 4T1-Ori and 4T1 #8 cells. 4T1-Ori or 4T1 #8 cells were injected into the mammary fat pads of BALB/s mice.

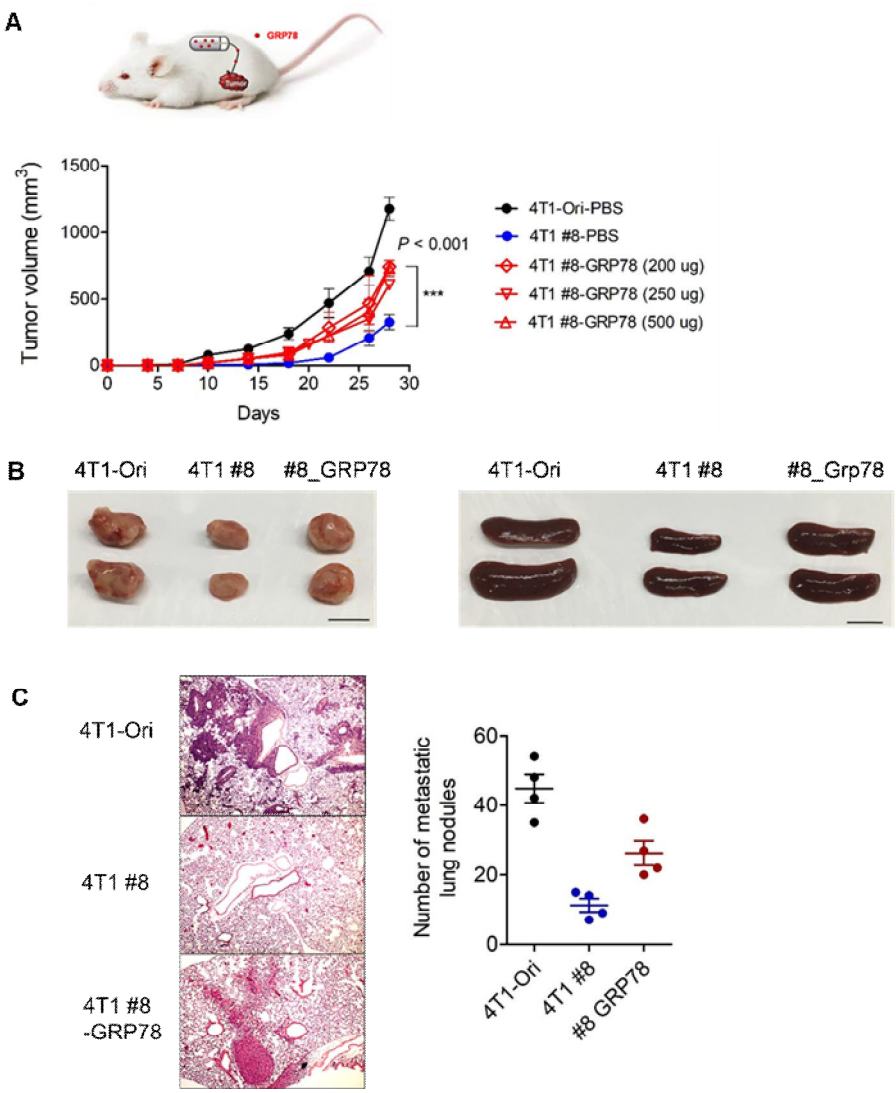
(A) Line graph of primary cancer growth (n=10).

(B) Representative hematoxylin and eosin (H&E) staining photograph of lungs at 21 days post-cancer injection. Number of metastatic nodules in spleens and lungs at 21 days.

(C) Percentages of MDSC (CD11b+ Gr-1+), CD4+ T cells, and CD8+ T cells at 21 days post-injection/total cells in cancer by flow cytometry.

(D) Absolute numbers of total cells, MDSC (CD11b+ Gr-1+), CD4+ T cells, and CD8+ T cells in the cancer at 21 days post injection (n=5).

Data are expressed as the mean ± SEM of each group. The p values were obtained using two-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



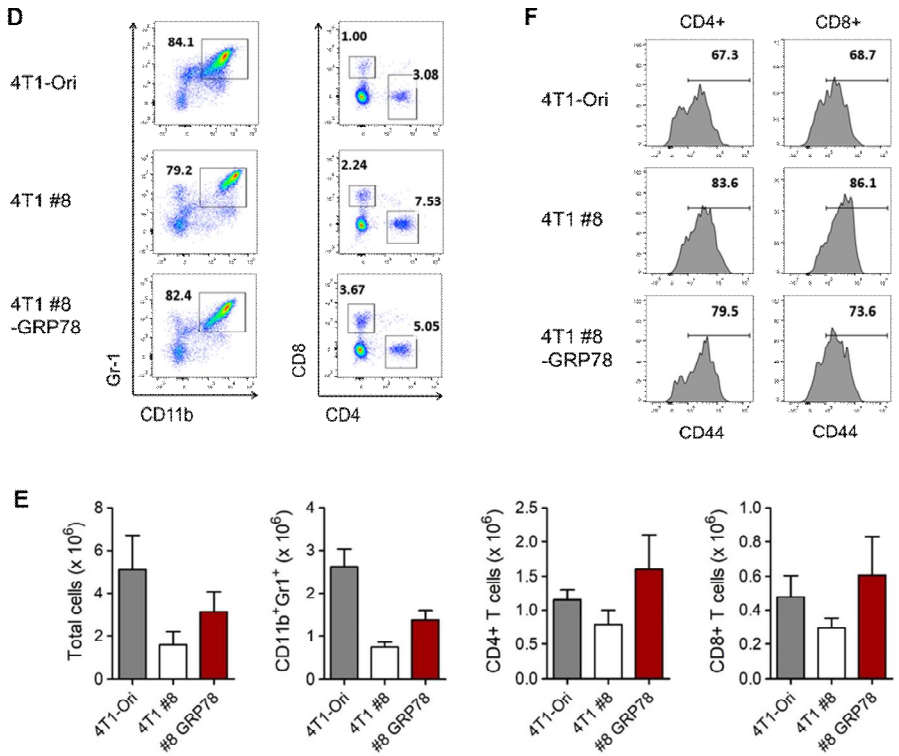



**Figure 10. Restoration of cancer growth by induction of GRP78 *in vivo***

The line graph shows the *in vivo* cancer growth of 4T1 #8 cells with induction of GRP78. 4T1 #8 cells were injected into the mammary fat pads of BALB/c mice. An Alzet osmotic pump on the back of mice for 28 days released 0.25 µl/hr of recombinant GRP78 to the cancer site.

(A) Primary cancer growth (n=4).

(B) Representative photograph of cancers and spleen at 28 days.

(C) Representative hematoxylin and eosin (H&E) staining photograph of lungs at 28 days post-injection. Number of metastatic nodules in spleens and lungs at 28 days.

(D) The percentages of MDSC (CD11b+ Gr-1+), CD4+ T cells, and CD8+ T cells at 21 days/total cells in the cancer by flow cytometry.

(E) Absolute numbers of total cells, MDSC (CD11b+ Gr-1+), CD4+ T cells, and CD8+ T cells in the cancer at 21 days post-injection (n=5).

(F) CD44 detection of CD4+ T cells and CD8+ T cells at 28 days post-injection by flow cytometry.

Data are expressed as the mean ± SEM of each group. The p values were obtained using two-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

# DISCUSSION

Cancers develop a cancer microenvironment to promote survival and increase the tumorigenesis of cancer cells [1]. The cancer microenvironment is composed of various factors released from both cancer and non-cancer cells. GRP78 is a chaperone protein that belongs to the heat shock protein (HSP) 70 family and is present in the cancer microenvironment [13]. In addition, GRP78 protein levels increase due to stress in the ER and cytoplasm, which can result in additional GRP78 secretion into the microenvironment [20,21].

In this study, I showed that GRP78 is a major factor responsible for activating MDSCs, thus inducing IL-6 expression and increasing the favorability of the cancer microenvironment towards metastasis in 4T1 cells (a metastatic breast cancer cell line) (Fig. 1). GRP78 protein was added to 4T1-conditionded medium before being added to MDSCs, and was found to increase IL-6 production and MDSC activation compared to 4TI-conditioned medium alone. Moreover, gene ontology examination of GRP78-tretead splenic MDSCs showed that MDSC functionally differentiated in the presence of GRP78 (Fig. 3). However, additional studies are needed to clarify the phenotype, differentiation, and effect on angiogenesis of GRP78-treated MDSCs.

GRP78 induces ER stress in MDSCs (Fig. 4), and appropriate ER stress facilitates the survival and proliferation of MDSCs in the cancer-environment. Therefore, ER stress signaling may be the first signal for MDSC activation. In addition, GRP78 has been shown to chronically activate immune pathways and the innate immune response (Fig. 3). In this study, I showed that GRP78 up-regulates PD-L1 on MDSCs, but does not affect the expression of PD-L2 on splenic MDSCs (Fig. 5). This may be because the expression of the two PD-1 ligands differs among

cell types. PD-L1 is highly expressed on the surface of T and B cells, dendritic cells, monocytes, MDSCs, and cancer cells. In contrast, PD-L2 is expressed on the surface of activated dendritic cells and monocytes. Previous studies have shown that MDSCs regulate T cell activity in the cancer microenvironment through several pathways, including exhaustion and anergy of T cells, induction of T cell apoptosis, and generation of regulatory T cells. I showed that GRP78 can suppress CD8+ T cell proliferation. HAVCR2 and PDCD1 genes were also found to be upregulated in GRP78-treated splenic MDSCs in the ClueGo functional annotation chart, so I wonder the relation of immune check point molecule genes on MDSCs. Additional studies are needed to determine the how influence HAVCR2 and PDCD1 genes as the immune check point molecules on MDSCs and how GRP78 affects the expression of MHCII on MDSCs as the antigen-presenting cells (APCs). In addition, I could suggest the expression of ADAM17 on GRP78-treated splenic MDCSs facilitates not only the migration of cancer cells but also the release of the soluble IL-6Rα. Therefore, GRP78 contributes to the invasion of cancer cells and distant metastasis mediated MDSCs through IL-6 trans-signaling (Fig. 6).

Secreted GRP78 proteins influence monocyte differentiation by binding to Toll-like receptor-2 (TLR2), TLR4, CD14, and CD91. In this study, many functional pathways identified through the ClueGo oncology examination were related to TLR signaling. [17,19] GRP78 treatment of murine myeloid CD11c+ cells induced a tolerogenic phenotype that was comparable to immature DCs [23]. MDSCs are immature myeloid cells that can differentiate into other myeloid cells. [26]. According to ClueGo functional annotation chart, there were many genes related to macrophage, so it could be related to MDSC differentiation to other myeloid cells. Therefore, MDSCs are thought to confer a microenvironment favorable to cancers due to their ability to suppress innate and adaptive immunity

[13,14].

To verify the role of GRP78 released from cancer cells on MDSCs *in vivo*, 4T1 #8 cells expressing low levels of GRP78 were compared to original 4T1 cells to evaluate the connection between cancer-derived GRP78 and MDSCs in the cancer-environment (Fig. 9). It was difficult to establish stable shGRP78 and GRP78-overexpressing cancer cell lines, since those cell lines were not be maintain and induced death. The over expressing GRP78 may induce apoptosis over stressful conditions, and deletion of GRP78 induce activation of PERK, ATF6 and IRE1 by detaching GRP78 and unfolded protein response to cell death. GRP78 could play a significant role in the survival of cancer cells. Therefore, I ensured that the 4T1 #8 cell lines with low expressing GRP78 could be maintained a consistent cell type and expressed consistent levels of GRP78.

I verified that the down-regulated GRP78 cell line did not build up cancer sphere and develop drug resistance (Fig. 8). These findings support the notion that the 4T1 #8 cell line expresses appropriately low levels of GRP78, and that GRP78 affects both cancer cells and MDSCs in the cancer microenvironment. Together, these *in vivo* results indicate that adding GRP78 to a cancer as a soluble factor may restore cancer growth and metastasis due to the induction of ER stress and T cell suppression by activated MDSCs.

In conclusion, this study showed that GRP78, the main mediator of ER stress, influences the metastatic cancer microenvironment, and particularly the anti-cancer immune response. In addition, extracellular GRP78 can affect the anti-cancer response by increasing regulation of the cancer microenvironment. Therefore, GRP78 may have potential therapeutic benefits due to its ability to influence cancer cells and other cells in the cancer microenvironment.

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# 국문 초록

암은 우리 몸에 생긴 새롭고 비정상적인 기관이다. 암세포는 종양을 형성하고 살아님기 위해서 자신에게 유리한 종양미세환경을 만든다. 암세포가 분비하는 물질은 종양미세환경에 존재하는 면역세포들을 포함한 다른 세포들을 조절하고, 세포들 간의 상호 관계는 종양 형성과 전이뿐만 아니라 면역억제반응 등 다양한 면에서 매우 중요하다. 이전 연구에서 interleukin-6 (IL-6)를 높게 발현하는 전이성 유방암 세포가 골수유래면역억제세포를 종양미세환경으로 불러오며, 골수유래면역억제세포와의 IL-6 트랜스 신호전달을 통해 유방암세포의 침투성과 전이를 촉진시킨다는 메커니즘을 확인하였다. IL-6를 통해 비전이성 유방암세포가 종양미세환경으로 골수유래면역억제세포를 유도할 수 있었지만, 암 전이는 크게 발생하지 못하였다. 이를 통해 암세포에서 분비한 어떠한 물질이 종양미세환경에서 불려온 골수유래면역억제세포를 활성화 시키고 암 전이를 유도하는지 의문이 생겼다.

이번 연구에서는 전이성 유방암 세포는 78kD glucose-regulated protein 78 (GRP78)을 높이 발현하고 종양미세환경으로 분비하며, 골수유래면역억제세포의 IL-6 생산 및 활성화를 이끌어 전이성 암 진행을 조절하는 요소임을 밝혔다. 암세포에서 분비된 GRP78은 골수유래면역억제세포에 ER 스트레스를 발생시켜 종양미세환경에서 살아남을 수 있게 하며, PD-L1을 발현하고 IL-10을 분비해 T 세포를 억제하는 면역억제작용을 강화시킨다. 또한 ADAM17을 발현하여 암 세포의 침투와 이동을 용이하게 도와준다. 암세포 내의 GRP78은 직접적으로 암세포의 형성과 침투성 그리고 약 내성에 영향을 미치며, MDSC를 조절하여 종양형성과 전이에 큰 역할을 한다. 요약하자면, 전이성 유방암세포는 높은 GRP78을 발현하여 암줄기세포 성격을 유지하면서 증가된 GRP78을

세포 밖 종양미세환경으로 분비하여 직접적으로 골수유래면역억제세포를 활성화시켜 암세포의의 종양 형성과 전이를 이끈다. 이는 GRP78를 암세포의 새로운 치료 타켓으로 전이성 암 진행과정을 막는 새로운 방향을 제시하였다.

주요어: 유방암 세포, 골수유래면역억제세포, glucose-regulated protein 78, 암 전이, 종양미세환경, ER스트레스, 면역억제
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