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TLR-Mediated Secretion of Endoplasmic Reticulum Aminopeptidase 1 from Macrophages

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Macrophages play an important role in host defense under several immunological, inflammatory, and/or infectious conditions. In our previous work, we demonstrated that endoplasmic reticulum aminopeptidase 1 (ERAP1) was secreted from macrophages in response to LPS and IFN-γ, and it enhanced their phagocytic activity. In this study, we analyzed the mechanism of LPS/IFN-γ–induced ERAP1 secretion. LPS/IFN-γ–induced secretion of the enzyme from the murine macrophage cell line RAW264.7 was suppressed by polymyxin B. Several agonists of TLRs, such as Pam3CSK4, FSL-1, and ODN1826, induced its secretion. In contrast, neutralizing Abs to IFN-β and TNF-α receptor type 1 suppressed its secretion. Using murine peritoneal macrophages derived from TNF-α and type 1 IFNRI knockout mice, we confirmed the involvement of these two cytokines in ERAP1 secretion. In addition, secretion of ERAP1 from both RAW264.7 cells and murine peritoneal macrophages was induced by A23187 and thapsigargin and inhibited by BAPTA-AM and the calmodulin inhibitor W7. These results suggest that LPS/IFN-γ–induced secretion of ERAP1 is mediated by TLRs via induction of intermediate cytokines such as IFN-β and TNF-α, which in turn lead to enhanced cytosolic Ca2+ levels and calmodulin activation. The Journal of Immunology, 2014, 192: 000–000.

Endoplasmic reticulum aminopeptidase 1 (ERAP1) is well established as a multifunctional enzyme belonging to the M1 family of aminopeptidases, with roles in blood pressure regulation, angiogenesis, ectodomain shedding of several cytokine receptors, and processing of antigenic peptides presented to MHC class I molecules (1–4). Its cDNA was initially cloned as an analog of angiogenesis (9, 10). In addition, it was also reported that ERAP1/puromycin-insensitive leucyl-specific aminopeptidase was previously reported that the enzyme is localized in the ER and acts of obvious ER retention signals in the molecule. In contrast, M1 aminopeptidases” (1, 5). Because of its unique role in Ag presentation in the ER, this enzyme is currently designated as ERAP1.

Although ERAP1 plays important roles in several pathophysiological processes, its subcellular localization, on the basis of the available reports, remains debatable. We and others (6–8) have previously reported that the enzyme is localized in the ER and acts as a final processing enzyme of antigenic peptides, despite the lack of obvious ER retention signals in the molecule. In contrast, ERAP1/puromycin-insensitive leucyl-specific aminopeptidase was reported to be localized in the cytosol, playing roles in the regulation of angiogenesis (9, 10). In addition, it was also reported that ERAP1/aminopeptidase regulator of TNF-α receptor type 1 (TNFR1) shedding was a type II integral membrane protein, which binds to cytokine receptors such as TNF type 1, IL-1 type II, and IL-6α and promotes their ectodomain shedding (11–13). ERAP1 may change its subcellular localization depending on the cell type expressing the enzyme or the environmental changes that the cells are exposed to. ERAP1 may exert its multifunctional properties by changing its localization.

Activated macrophages play a key role in host defense and tissue repair after injury by inducing several proinflammatory cytokines (14). In our previous work, we reported that LPS and IFN-γ synergistically induced ERAP1 secretion from the ER of the murine macrophage cell line RAW264.7, which suggested the involvement of TLR4 in ERAP1 secretion (15). LPS/IFN-γ treatment led to enhancement of the FcR-dependent phagocytic activity of the cells, which was found to be sensitive to the aminopeptidase inhibitor amastatin. Moreover, direct addition of ERAP1 to the culture medium of RAW264.7 cells also enhanced their phagocytic activity. Thus, our data strongly suggested that aminopeptidases were involved in macrophage activation and that ERAP1 functioned as a stimulator of macrophage phagocytic activity.

In this study, we further analyzed the LPS/IFN-γ–induced secretion of ERAP1 from RAW264.7 cells and found that induction of proinflammatory cytokines such as IFN-β and TNF-α via the TLR-mediated signaling pathway is crucial in the LPS/IFN-γ–induced secretion of ERAP1. Further, induced cytokines in turn mediated the increase in cytoplasmic Ca2+ levels, which ultimately led to induction of ERAP1 secretion through calmodulin activation. The data presented in this study, which showed a regulated secretion of ERAP1 through the immune system under certain inflammatory/infectious conditions, might provide new insights into the pathophysiological significance of the enzyme in host defense.

Materials and Methods

Expression and purification of recombinant ERAP1
S9 insect cells were transfected with bacmid DNA by using the Cellfectin reagent (Invitrogen, Carlsbad, CA), and recombinant baculoviruses were harvested after 72 h of incubation. For the expression of recombinant
ERAP1, S9 cells (2.0 × 10^6 cells/ml) infected with the recombinant baculovirus (multiplicity of infection of 1–3) were cultured for 72 h in 100 ml SF-900 III medium (Invitrogen) at 27˚C.

Culture medium containing recombinant ERAP1 was collected by centrifugation and loaded onto a hydroxypatate (Nacalai Tesque, Kyoto, Japan) column (bed volume 10 ml) pre-equilibrated with 5 mM phosphate buffer (pH 7.5) and eluted with 100 mM phosphate buffer (pH 7.5). The eluate was applied to a Co2+-chelating Sepharose column (bed volume 1 ml) (GE Healthcare) pre-equilibrated with 10 mM phosphate buffer containing 0.1 M NaCl and then eluted with 150 mM imidazole. ERAP1-containing fractions were extensively dialyzed against 25 mM Tris/HCl buffer (pH 7.5), containing 0.125 M NaCl, concentrated with an ultrafiltration membrane, and stored at −20˚C until use. After purification, ERAP1 was resolved as a single ~105-kDa band through SDS-PAGE.

**Generation of a stable reporter cell line**

A DNA fragment corresponding to the promoter region of the mouse IFN-β gene (from nucleotide position −490 to 55) was amplified by PCR from mouse genomic DNA using primers with XhoI and BglII sites (5’-ATCGAGATGGGGTTCTTCTCCTCA-3’ and 5’-GCAAGATCTGTCTCTCCTGAC-3’) and subcloned into the corresponding sites of the pGL4.10-luc2 plasmid (Promega). The firefly luciferase gene in the resulting plasmid was replaced by the codon-optimized Metridia luciferase (2 TLR-INDUCED SECRETION OF ERAP1

The BglII and BamHI sites (5’ and 3’ sites. A 1036-bp fragment containing the blasticidin resistance gene under the control of the SV40 promoter and synthetic polyadenylation signal was inserted into the multiple cloning site of the resulting plasmid to obtain the plasmid pGL4-mIFN-490/+55-MetLuc-Bla. The pGL4-mIFN-490/+55-MetLuc-Bla plasmid was linearized site of the resulting plasmid to obtain the plasmid pGL4-mIFN-490/+55-MetLuc-Bla. The pGL4-mIFN-490/+55-MetLuc-Bla plasmid was linearized by digestion with the Sall restriction enzyme and transfected into RAW264.7 cells by electroporation using the Nepa21 apparatus (Nepa Gene, Chiba, Japan). Stably transfected cells were selected by adding blasticidin (2 μg/ml) to the culture medium on the following day.

**Western blot analysis**

Secreted ERAP1, GAPDH, and TNF-α were detected by Western blot analysis. In brief, blots were probed with anti-ERAP1 Ab (6, 16), GAPDH Ab (Santa Cruz Biotechnology, Santa Cruz, CA), or TNF-α Ab (Santa Cruz Biotechnology), and the immune complexes were treated with HRP-labeled secondary Ab. The protein bands were visualized using a LAS-4000 mini luminous image analyzer (GE Healthcare, Buckinghamshire, U.K.) using the ECL prime Western blotting detection kit (GE Healthcare).

**IFN-β reporter assay**

MetLuc activity was measured using a Dual-Luciferase Reporter Assay System (Promega) according to the instruction manual. In brief, culture supernatant containing secreted MetLuc was mixed with coelenterazine so-solution. Chemiluminescence from coelenterazine was then detected using the multimicroplate reader MTP-810 Lab (Hitachi High Tech, Tokyo, Japan).

**IL-2 induction of secretion**

RAW264.7 cells (5 × 10^5 cells/ml) were plated on coverslips in a 24-well plate and treated with the respective stimulants. After stimulation, cells were washed once with cold PBS and fixed with cold methanol for 5 min at −25˚C. Cells were then permeabilized with 1% Triton X-100 in PBS (pH 7.4) for 10 min. The coverslips were blocked with 5% TBS containing 0.1% skim milk (blocking buffer) for 30 min at room temperature and incubated with the respective primary Abs in blocking buffer at 4˚C overnight. The Abs used were as follows: rabbit polyclonal anti-stromal interaction molecule 1 (STIM1) IgG (Sigma-Aldrich, St. Louis, MO), murine monoclonal anti-Annexin II (ANX II) IgG (BD Biosciences, San Jose, CA), and rabbit polyclonal anti-myosin L chain 2 (phospho-Ser18) IgG (Signalway Antibody, Pearlland, TX). The primary Abs were visualized using Alexa 488-conjugated anti-murine IgG or Alexa 594-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR). After unbound dyes were washed off with T-PBS, coverslips were mounted with PermaFluor (Thermo Fisher Scientific, Waltham, MA) on a glass slide. Images were captured by using an ECLIPSE 80i microscope (Nikon, Tokyo, Japan) with a ×40 objective using a 488- or 594-nm laser as excitation light source.

**Collecting macrophages from mice**

Animals were maintained under controlled air conditions (room temperature 24 ± 2˚C; and humidity 50 ± 10%) with food and water available ad libitum. Animals were housed under a 12-h light/dark cycle with a light intensity of 200–300 lux. All animal housing and experimental procedures were carried out in accordance with the guidelines of the Science Council of Japan and approved by the Institutional Animal Care and Use Committee of the Teikyo-Heisei University.

For collections of macrophages, mice were injected i.p. with 2 ml sterile 3% Brewer thioglycollate broth (Difco, Detroit, MI). After 4 d, animals were sacrificed, and thioglycollate-elicted peritoneal macrophages were obtained by lavage of the peritoneal cavity. Cells were seeded on 24-well plates (3 × 10^5 cells/well) and allowed to adhere to the tissue culture plates for 4 h at 37˚C. After gentle rinse to remove nonadherent cells, the adherent cells were collected and used as murine peritoneal macrophages.

**Cell culture**

The murine macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (Manassas, VA). RAW264.7 cells and murine peritoneal macrophages were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS. For assays, cells were transferred to and cultured in 24-well cell-culture plates (5 × 10^5 cells/well) and activated with various stimuli at 37˚C in serum-free RPMI 1640.

**Knockout mice**

ERAP1 knockout (ERAP1+/−; C57BL/6 background) mice (17) were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR4 knockout (TLR4−/−; BALB/c background) (18), Toll/IL-1R domain-containing adapter-inducing IFN-β (TRIF) knockout (TRIF−/−; C57BL/6 background) (19), and MyD88 knockout (MyD88−/−; BALB/c background) mice (20) were purchased from Oriental Kobo (Tokyo, Japan). Type 1 IFNR (IFNAR) knockout (IFNAR−/−; A129 background) (21) and TNF-α knockout (TNF-α−/−; BALB/c background) mice (22) were obtained from B&K Universal (Grimston, Aldbrough, U.K.) and RIKEN BioResource Center (Ibaraki, Japan), respectively.

**Materials**

RPMI 1640, polymyxin B, thapsigargin, forskolin, BAPTA-AM, and dibutyl cAMP were obtained from Nacalai Tesque (Kyoto, Japan). A23187, 2,5-di-tert-butylhydroquinone (DBHQ), Toll/IL-1R domain-containing adapter protein (TIRAP) inhibitory peptide, NF-κB activation inhibitor II, and W7 were from Merck (Whitehouse Station, NJ), LPS from Escherichia coli O55:B5 was obtained from Sigma-Aldrich (St. Louis, MO). Other TLR agonists were purchased from Invivogen (San Diego, CA). A neutralizing Ab against murine TNFR1 was purchased from R&D Systems (Minneapolis, MN). Neutralizing Abs against murine IFN-β and IL-1β were obtained from Yamasaki Shoya (Tokyo, Japan) and Abcam (Kentridge, U.K.), respectively. Mouse rIFN-β was purchased from PBL InterferonSource and mouse rTNF-α and rIFN-γ were purchased from PeproTech (Rocky Hill, NJ).

**Statistical analysis**

Data are presented as mean ± SD. Groups were compared by one-way ANOVA. The mean values of groups were compared separately using the Tukey–Kramer multiple comparison test.

**Results**

**Involvement of TLRs in the secretion of ERAP1 from RAW264.7 cells**

Fig. 1 confirms the specificity of anti-ERAP1 Ab employed in this study. Although cell lysate of macrophages obtained from wild-
and IFN-β manner. In addition, when suboptimal concentrations of TNFR1-neutralizing Abs suppressed ERAP1 secretion in a dose-dependent manner (23, 24). As shown in Fig. 3A, both TNFR1- and IFN-β are known to be secreted after stimulation of the MyD88-mediated secretion because several cytokines, including TNF-α, are known to be secreted in a dose-dependent manner. To examine whether TLR4, an LPS receptor, was involved in the secretion of ERAP1, we next measured the expression and secretion of TNF-α and IFN-β by stimulating RAW264.7 cells with various TLR agonists (Fig. 2B). LPS is known to be secreted in a dose-dependent manner. LPS/IFN-γ-induced secretion of ERAP1 was observed. In contrast, LPS enhanced IFN-β secretion, and IFN-β neutralizing Ab had little effect on the secretion, under experimental conditions. As expected, polymyxin B had little effect on the LPS/IFN-γ-induced secretion of ERAP1, even with polymyxin B (1 μg/ml) for 24 h. This result suggests that the MyD88-mediated signal delivered by TLR agonists activates the MyD88-dependent pathway, and that TLR4 is indeed involved in the LPS/IFN-γ-induced secretion of ERAP1. 

Next, we examined the roles of TLRs in ERAP1 secretion by using various TLR agonists (Fig. 2B). None of the agonists used in this study could induce ERAP1 secretion. In the presence of IFN-γ, the TLR agonists such as Pam3CSK4 (TLR1/2), FSL-1 (TLR2/6), and ODN1826 (TLR9) induced ERAP1 secretion in a dose-dependent manner. In contrast, polyinosinic-polycytidylic acid [poly(I:C), a TLR3 agonist] had no or little inducing activity up to 100 μg/ml, even in the presence of IFN-γ. These results suggest that each TLR type mediates distinct secretion signals of ERAP1.

TLRs mediate MyD88-dependent and -independent (i.e., TRIF-dependent) signals (23). Because the three agonists that induced ERAP1 secretion activate the MyD88-dependent pathway, the TLR-mediated secretion mechanism on promoter activity and IFN-β expression on the expected m.w. of ERAP1, no band was detected in cell lysate prepared from macrophages of ERAP1−/− mice, indicating that the Ab recognized ERAP1 exclusively. We then investigated whether TLR4, an LPS receptor, was involved in the secretion of ERAP1. As shown in Fig. 3A, both TNFR1- and IFN-β neutralizing Abs suppressed ERAP1 secretion in a dose-dependent manner. In addition, when suboptimal concentrations of TNFR1- and IFN-β-neutralizing Abs were used together, an additive suppressive effect on ERAP1 secretion was observed. In contrast, the IL-1β-neutralizing Ab had little effect on the secretion, under our experimental conditions. These results suggest that TNF-α and IFN-β, but not IL-1β, are involved in ERAP1 secretion.

FIGURE 1. Specific recognition of ERAP1 by anti-ERAP1 Ab employed in this study. (A) SDS-PAGE analyses of cell lysates of macrophages obtained from wild-type and ERAP1−/− mice. (B) Western blot analyses of cell lysates of macrophages obtained from wild-type and ERAP1−/− mice (top panel) detection of ERAP1, [bottom panel] detection of GAPDH as an internal control.

FIGURE 2. TLR-mediated secretion of ERAP1 from RAW264.7 cells. (A) Inhibition of LPS/IFN-γ-induced ERAP1 secretion from RAW264.7 cells by polymyxin B. RAW264.7 cells were treated with or without LPS (1 μg/ml)/IFN-γ (100 IU/ml) for 24 h at 37°C. Conditioned media were then collected, and secreted ERAP1 was detected by Western blot analysis. (B) Role of the TLR-signaling pathway on ERAP1 secretion from RAW264.7 cells in response to LPS/IFN-γ. RAW264.7 cells were treated with or without LPS (1 μg/ml)/IFN-γ (100 IU/ml) for 24 h at 37°C. Conditioned media were then collected, and secreted ERAP1 was detected by Western blot analysis. Conditioned media of RAW264.7 cells treated with LPS (1 μg/ml)/IFN-γ (100 IU/ml) were used as a positive control. (C) Roles of the TIRAP inhibitory peptide or NF-κB activation inhibitor II for 24 h at 37°C. Conditioned media were then collected, and secreted ERAP1 was detected by Western blot analysis.
When RAW264.7 cells were treated with TLR agonists, both Pam3CSK4 and poly(I:C) enhanced IFN-β transcription, irrespective of the presence of IFN-γ (Fig. 4D). Remarkably, poly(I:C), which did not induce ERAP1 secretion, enhanced IFN-β promoter activity. Because poly(I:C) is known to activate the MyD88-independent TRIF/TRIF-related adaptor molecule–mediated pathway, but not the MyD88-dependent pathway, to induce IFN-β (23), only induction/secretion of IFN-β may not be sufficient for ERAP1 secretion. In contrast, whereas TNF-α secretion was induced by Pam3CSK4 treatment as expected, poly(I:C) had no effect on its secretion (Fig. 4E). Considering that both anti–IFN-β and anti–TNFR1 Abs suppressed ERAP1 secretion from RAW264.7 cells, these results suggest that both IFN-β and TNF-α are required for ERAP1 secretion.

To examine this hypothesis, we analyzed the cytokine-induced secretion of ERAP1. RAW264.7 cells were treated with various combinations of IFN-β, TNF-α, and IFN-γ (Fig. 4F). None of the cytokines tested had an effect on ERAP1 secretion when used alone. Any combinations of the two cytokines tested had no or weak effects. However, when cells were treated with all three cytokines together, significant secretion of ERAP1 was observed. These results indicate that in addition to IFN-γ, both IFN-β and TNF-α are required for maximal ERAP1 secretion.

**Role of intracellular Ca²⁺ movement in the secretion of ERAP1 from RAW264.7 cells**

Cytokines often induce intracellular Ca²⁺ movement (25–27); hence, we next examined the role of intracellular Ca²⁺ in the secretion of ERAP1. For this purpose, we examined the effects of Ca²⁺ modulators that enhance intracellular Ca²⁺ levels. As shown in Fig. 5A, A23187, a Ca²⁺ ionophore, and thapsigargin, an inhibitor of the sarcoplasmic/ER Ca²⁺-ATPase to increase the intracellular Ca²⁺ level, induced ERAP1 secretion from RAW264.7 cells. However, DBHQ, a weaker inhibitor of the sarcoplasmic/ER Ca²⁺-ATPase, had little effect on the secretion, even at a concentration of 50 μM. In contrast, reagents known to enhance intracellular cAMP levels did not induce ERAP1 secretion. These results suggest that increase in the cytoplasmic Ca²⁺ level is important for the secretion of ERAP1 from RAW264.7 cells. Fig. 5B shows the dose-dependent induction of ERAP1 secretion from RAW264.7 cells by increasing intracellular Ca²⁺ levels.
secretion from RAW264.7 cells by thapsigargin. Notably, at a concentration of 5 μM, a 105-kDa band was observed in the culture supernatant. Because this band contained endoglycosidase H–sensitive sugar chains, this protein was most likely released directly from dead cells during incubation (15). In fact, the appearance of dead cells was apparent under the microscope in the presence of 5 μM thapsigargin; therefore, we used 2 μM thapsigargin for further analyses. Time-course experiments showed that ERAP1 secretion was first observed as early as 4 h after treatment with 2 μM thapsigargin, and the lag time in this case was much shorter than that in the LPS/IFN-γ–mediated secretion (Fig. 5C).

We next examined the effects of TIRAP inhibitory peptide and NF-κB activation inhibitor II on thapsigargin-induced ERAP1 secretion (Fig. 5D). Although the concentrations used in this study were the same as those presented in Fig. 2C, both inhibitors showed little inhibitory effects as expected.

To further elucidate the role of elevation of cytosolic Ca2+ level in ERAP1 secretion from RAW264.7 cells, we examined the effect of BAPTA-AM, a membrane-permeable Ca2+ chelator in cells, on ERAP1 secretion induced by several stimuli. As shown in Fig. 6A, BAPTA-AM inhibited LPS/IFN-γ– and thapsigargin–induced ERAP1 secretion, further supporting that elevation of intracellular Ca2+ levels plays a role in the secretion.

To visualize intracellular Ca2+ mobilization in RAW264.7 cells, redistribution of a Ca2+ sensor STIM1 from the ER to the cell membrane was monitored using ANX II as a cell-membrane

FIGURE 4. Involvement of TNF-α and IFN-β in LPS/IFN-γ–induced ERAP1 secretion from RAW264.7 cells. (A) Enhancement of transcription and secretion of IFN-β by TLR4 activation from RAW264.7 cells. RAW264.7 cells or RAW264.7 cells stably transfected with the IFN-β promoter region fused with the MetLuc gene (IFNB-MetLuc RAW) were treated with or without LPS (1 μg/ml) and/or IFN-γ (100 IU/ml) for 24 h at 37˚C. Conditioned media were then collected, and secreted IFN-β and MetLuc activities were measured as described in the Materials and Methods. Luciferase activity in the culture medium of LPS/IFN-γ–stimulated RAW264.7 cells was taken as 100% (left panel). Data are expressed as the mean ± SD (n = 3). (B) Time course of LPS/IFN-γ–induced transcription and secretion of IFN-β. RAW264.7 cells or IFNB-MetLuc RAW cells were treated with LPS (1 μg/ml)/IFN-γ (100 IU/ml) for the indicated times at 37˚C, and transcription and secretion of IFN-β were measured using luciferase assay and ELISA, respectively. Luciferase activity in the culture medium of LPS/IFN-γ–stimulated RAW264.7 cells was taken as 100% (left panel). Data are expressed as the mean ± SD (n = 3). (C) Secretion of TNF-α by LPS and IFN-γ (top panel) RAW264.7 cells were treated with or without LPS (1 μg/ml) and/or IFN-γ (100 IU/ml) for 24 h at 37˚C, [bottom panel] RAW264.7 cells were treated with or without LPS [1 μg/ml]/IFN-γ [100 IU/ml] for the indicated times at 37˚C. Conditioned media were then collected, and secreted TNF-α was detected by Western blot analysis. (D) Enhancement of IFN-β promoter activity by TLR agonists. IFNB-MetLuc RAW cells were treated with or without Pam3CSK4 (1 μg/ml) or poly(I:C) (100 μg/ml) in the presence or absence of IFN-γ (100 IU/ml) for 24 h at 37˚C. Conditioned media were then collected, and secreted MetLuc activity was measured as described in the Materials and Methods. Luciferase activity in the culture medium of LPS (1 μg/ml)/IFN-γ–stimulated (100 IU/ml) RAW264.7 cells was taken as 100%. Data are expressed as the mean ± SD (n = 3). (E) Enhancement of TNF-α expression by TLR agonists. RAW264.7 cells were treated with or without Pam3CSK4 (1 μg/ml) (left panel) or poly(I:C) (100 μg/ml) (right panel) in the presence or absence of IFN-γ (100 IU/ml) for 24 h at 37˚C. Conditioned media of RAW264.7 cells treated with LPS (1 μg/ml)/IFN-γ (100 IU/ml) used as a positive control. Conditioned media were then collected, and secreted TNF-α was detected by Western blot analysis. (F) Effects of various combinations of cytokines on ERAP1 secretion from RAW264.1 cells. RAW264.7 cells were treated with various combinations of TNF-α (10,000 IU/ml), IFN-β (10,000 IU/ml), and IFN-γ (1,000 IU/ml) for 24 h at 37˚C. Conditioned media of RAW264.7 cells treated with LPS (1 μg/ml)/IFN-γ (100 IU/ml) used as a positive control. Conditioned media were then collected, and secreted ERAP1 was detected by Western blot analysis. *p < 0.05, **p < 0.01, ***p < 0.001.
secretion is mainly regulated by influx of extracellular Ca^{2+}.

These results suggest that ERAP1 secretion from RAW264.7 cells is regulated by influx of extracellular Ca^{2+} through store-operated Ca^{2+} channels, including STIM1-dependent Ca^{2+} channels (29, 30).

**Involved of calmodulin in the mechanism of ERAP1 secretion**

Elevation of intracellular Ca^{2+} levels causes calmodulin activation, which is critical in various signal transduction systems (27, 31–33); therefore, we next examined the correlation between calmodulin activation and ERAP1 secretion to test the possible involvement of calmodulin in the secretion process. As shown in Fig. 7, all stimulants that induced ERAP1 secretion from RAW264.7 cells caused calmodulin activation, as determined from the phosphorylation of myosin L chain 2 (Ser^{18}). Importantly, treatment with W7, a calmodulin antagonist, inhibited both phosphorylation of the myosin L chain 2 and secretion of ERAP1, suggesting that calmodulin activation induced by a stimulus-dependent increase in the intracellular Ca^{2+} level was involved in the process of ERAP1 secretion.

**Secretion of ERAP1 from murine peritoneal macrophages**

To confirm the involvement of cytokines and intracellular Ca^{2+} movement in the secretion of ERAP1, we next used thiglycollate-elicted murine peritoneal macrophages. As shown previously (15), LPS induced the secretion of ERAP1 from peritoneal macrophages...
addition, secretion of the enzyme prepared from RAW264.7 cells. RAW264.7 cells were treated with or without LPS (1 μg/ml)/IFN-γ (100 IU/ml), Pam3CSK4 (1 μg/ml)/IFN-γ (100 IU/ml), TNF-α (10,000 IU/ml)/IFN-β (10,000 IU/ml)/IFN-γ (1,000 IU/ml), or thapsigargin (2 μM) for 24 h (LPS/IFN-γ, Pam3CSK4/IFN-γ, and TNF-α/IFN-β/IFN-γ) or 4 h (thapsigargin) at 37 °C in the presence or absence of 10 μM W7. After incubation, conditioned media were collected, and secreted ERAP1 was detected by Western blot analysis. Calmodulin activity was monitored by measuring the phosphorylation of myosin light chain 2 as described in the Materials and Methods. Scale bar, 10 μm.

**FIGURE 7.** The calmodulin antagonist W7 inhibits the stimulus-dependent secretion of ERAP1 from RAW264.7 cells. RAW264.7 cells were treated with or without LPS (1 μg/ml)/IFN-γ (100 IU/ml), Pam3CSK4 (1 μg/ml)/IFN-γ (100 IU/ml), TNF-α (10,000 IU/ml)/IFN-β (10,000 IU/ml)/IFN-γ (1,000 IU/ml), or thapsigargin (2 μM) for 24 h (LPS/IFN-γ, Pam3CSK4/IFN-γ, and TNF-α/IFN-β/IFN-γ) or 4 h (thapsigargin) at 37 °C in the presence or absence of 10 μM W7. After incubation, conditioned media were collected, and secreted ERAP1 was detected by Western blot analysis. (A) Effects of TLR agonists on ERAP1 secretion. Murine peritoneal macrophages were treated with or without Pam3CSK4 (1 μg/ml), poly(I:C) (100 μg/ml), and LPS (1 μg/ml) in the presence or absence of IFN-γ (100 IU/ml) for 48 h at 37 °C. Conditioned media were then collected, and secreted ERAP1 was detected by Western blot analysis. (B) Secretion of ERAP1 from peritoneal macrophages obtained from TLR4−/− mice. Peritoneal macrophages derived from wild-type (WT) and TLR4−/− mice were treated with or without (−) LPS (1 μg/ml) for 48 h at 37 °C. (C) Secretion of ERAP1 from peritoneal macrophages obtained from MyD88−/− (left panel) and TRIF−/− (right panel) mice. Peritoneal macrophages derived from WT and either MyD88−/− or TRIF−/− mice were treated with or without (−) LPS (1 μg/ml) for 48 h at 37 °C. (D) Secretion of ERAP1 from peritoneal macrophages obtained from TNF-α−/− mice. Peritoneal macrophages derived from WT and TNF-α−/− mice were treated with or without (−) LPS (1 μg/ml) and TNF-α (10,000 IU/ml) for 48 h at 37 °C. Conditioned media were then collected, and secreted ERAP1 was detected by Western blot analysis. (E) Secretion of ERAP1 from peritoneal macrophages obtained from IFNAR−/− mice. Peritoneal macrophages from WT and IFNAR−/− mice were treated with LPS (1 μg/ml) for 48 h at 37 °C. Conditioned media were then collected, and secreted ERAP1 was detected by Western blot analysis. (F) Effects of various combinations of cytokines on ERAP1 secretion from murine peritoneal macrophages. Murine peritoneal macrophages were treated with various combinations of TNF-α (10,000 IU/ml), IFN-β (2,500 IU/ml), and IFN-γ (1,000 IU/ml) for 48 h at 37 °C. Conditioned media of peritoneal macrophages treated with LPS (1 μg/ml) used as a positive control. Conditioned media were then collected, and secreted ERAP1 was detected by Western blot analysis. (G) Secretion of ERAP1 by reagents for Ca2+ mobilization. Murine peritoneal macrophages were treated with various reagents for 8 h at 37 °C. Secreted ERAP1 was then detected by Western blot analysis. (H) Effects of BAPTA-AM and W7 on ERAP1 secretion. Murine peritoneal macrophages were treated with 1 μg/ml LPS for 48 h at 37 °C in the presence of either 50 μM BAPTA-AM or 10 μM W7. Secreted ERAP1 was then detected by Western blot analysis.
indicate that IFN-β and TNF-α are required for ERAP1 secretion from murine peritoneal macrophages.

We then examined the effect of various combinations of the three cytokines on ERAP1 secretion from peritoneal macrophages (Fig. 8F). Treatments with suboptimal concentrations of each cytokine alone had little effect on the secretion. In contrast, when cells were treated with various combinations of the cytokines, all tested combinations induced significant secretion of the enzyme, indicating that any two cytokines acted on the cells cooperatively to induce ERAP1 secretion.

Fig. 8G shows the effects of various stimulants on the secretion of ERAP1 from murine peritoneal macrophages. As in the case of RAW264.7 cells, A23187 and thapsigargin induced secretion of ERAP1, as did DBHQ. We assumed in the previous work that peritoneal macrophages were already primed with IFN-γ, and thus, induced the secretion without IFN-γ (15, 34). Different priming stages of RAW264.7 cells and thiglycollate-elicited murine peritoneal macrophages might account for the differential sensitivity to a weaker inhibitor of sarcoplasmic/ER Ca2+-ATPase, DBHQ. As shown in Fig. 8H, LPS-induced secretion of ERAP1 was suppressed by BAPTA-AM and W7. Although W7 (10 μM) suppressed the secretion only partially, these results indicate the involvement of intracellular Ca2+ mobilization and the subsequent activation of calmodulin in the secretion of ERAP1 from peritoneal macrophages (Fig. 9).

Discussion

Although ERAP1 has no obvious ER retention signals in its primary structure, it is retained in the ER and acts as a final processing enzyme of antigenic peptides presented to MHC class I molecules (5–8). In contrast, we have previously reported that ERAP1 is secreted from macrophages in response to LPS/IFN-γ treatment and that it enhanced their phagocytic activity (15). In this study, we examined the secretion signals of ERAP1 from macrophages and found that TLR4 played a critical role in the LPS/IFN-γ-induced secretion of ERAP1 from RAW264.7 cells. Together with IFN-γ, IFN-β and TNF-α induced by TLR ligands mediated the increase in the intracellular Ca2+ level, thereby inducing ERAP1 secretion via calmodulin activation. Induction of several proinflammatory cytokines, including IFN-β, IFN-γ, and TNF-α, which were evaluated examined in this study, is an important mechanism through which macrophages control inflammatory and/or infectious responses (14, 21, 22). Therefore, our data may reveal the complexity of regulated secretion of ERAP1 for activation of macrophages in specific inflamed and/or infected sections, where the cytokines are induced and/or the cells are exposed to LPS.

LPS/IFN-γ–induced secretion of ERAP1 from RAW264.7 cells was considerably suppressed by polymyxin B, suggesting that signaling via TLR4 is critical for secretion. By using agonists of various TLRs, we also observed that TLRs interacting with the MyD88 could mediate ERAP1 secretion. TLR signaling uses two distinct adaptor proteins, MyD88 and TRIF (23). Although the MyD88-dependent pathway is known to mediate the induction of TNF-α and IFN-γ, TRIF has been shown to mediate IFN-β production through IFN regulatory factor (IRF) 3 (23). Our data employing MyD88−/− mice revealed that the MyD88 is the main signaling molecule required for the induction of ERAP1 secretion via TLR4 activation. It is conceivable that although MyD88 contributes to ERAP1 secretion through induction of TNF-α and IFN-β and is indispensable to the secretion, contribution of TRIF to the secretion is solely dependent on its ability to induce IFN-β (23, 24). Considering the synergistic induction of ERAP1 secretion from RAW264.7 cells by LPS and IFN-γ, the IFN-γ–mediated signaling pathway that primed macrophages could also be indispensable to the maximal response.

In this study, we made three separate observations supporting the role of Ca2+/calmodulin in LPS/IFN-γ–induced secretion of ERAP1 from RAW264.7 cells. First, A23187 and thapsigargin, both of which increase cytoplasmic Ca2+ levels, induced ERAP1 secretion. Second, BAPTA-AM, a blocker of intracellular Ca2+ mobilization, inhibited the stimulus-dependent secretion of the enzyme. Third, the calmodulin antagonist W7 inhibited the stimulus-dependent secretion of the enzyme. Because increase in the Ca2+ level in the cytosol was sufficient for ERAP1 secretion, cross-talk at the level of cytokine signaling pathways might contribute toward optimal Ca2+ entry into the cytosol.

Fig. 9 shows the possible scheme of the LPS/IFN-γ–induced ERAP1 secretion process from macrophages based on the results of this study. Signals delivered from the LPS receptor (TLR4) and IFN-γ receptor (IFNGR) activate transcription factors such as NF-kB, IRF3, and IRF1 to induce secretion of TNF-α and IFN-β (23, 35, 36). Subsequently, these three cytokines synergistically increase the intracellular Ca2+ level and activate calmodulin. Activated calmodulin might then enhance the expression of genes involved in ERAP1 secretion, presumably through activation of transcription factors such as CREB and NFAT (37). Mutual induction of these cytokines might be attributable to a positive-feedback loop for induction of ERAP1 secretion.

In our previous work, we reported that the exon 10 coding sequence of ERAP1 was critical for its ER retention (38). Certain proteins binding to the exon 10 sequence might be required for the ER retention of ERAP1. Saturation of the interaction between ERAP1 and putative binding protein may cause secretion of the enzyme. It is also possible that an ER protein that competes with the proteins binding to the exon 10 sequence of ERAP1 may disrupt the interaction in the ER to induce the secretion. Although several proteins have been identified as ERAP1-binding proteins, none of these is localized in the ER (11–13, 39–41). In our preliminary results, several proteins were observed to bind to the exon 10 sequence, few of which may act as competitors of the enzyme. To elucidate the ER retention and secretion mechanisms of ERAP1, identification and characterization of the proteins that bind to the exon 10 coding sequence of the enzyme in the ER are required.

Previous studies using aminopeptidase inhibitors have suggested that aminopeptidases are involved in the activation of macrophages (42, 43). We have previously reported that secreted ERAP1 directly

![FIGURE 9. Schematic representation of the ERAP1 secretion process from macrophages induced by TLR activation. Signals delivered from MyD88-dependent signaling via TLRs induce and secrete TNF-α and IFN-β. TNF-α and IFN-β then increase intracellular Ca2+ levels in the presence of IFN-γ and subsequently activate calmodulin, which induces ERAP1 secretion from macrophages.](http://www.jimmunol.org/content/1/1/726)
enlarged the phagocytic activity of both RAW264.7 cells and murine peritoneal macrophages, and we were the first, to our knowledge, to identify the aminopeptidase with macrophage-activating activity as ERAP1. Considering that IFN-β, IFN-γ, and LPS are indicators and/or modulators of either viral or bacterial infection in the body, secreted ERAP1 with phagocytosis-enhancing activity might play a role in host defense under several infectious conditions.

Until now, ERAP1 was reported to be involved in the pathogenesis of various diseases. Initially, ERAP1 was speculated to regulate blood pressure because it inactivates angiotensin II and converts kallidin to bradykinin (16). Subsequently, by screening for polymorphisms in the human ERAP1 gene, an association of the K282R variant of the enzyme with essential hypertension was reported (44). Indeed, the enzymatic activity of the variant was shown to be less than that of the wild-type, as determined using synthetic and natural peptide substrates (45, 46). The data presented in this study show secretion of ERAP1 under specific conditions and may provide a molecular basis of ERAP1-mediated blood pressure regulation in blood vessels by processing angiotensins II and III. In contrast, genome-wide association studies shown to be less than that of the wild-type, as determined using blood pressure regulation in blood vessels by processing angiotensins II and III. In contrast, genome-wide association studies indicated that ERAP1 was associated with several inflammatory diseases including ankylosing spondylitis, psoriasis, and Behçet disease (47–49). In these studies, interactions between ERAP1 and MHC class I molecules were observed, suggesting that aberrant disease (47–49). In these studies, interactions between ERAP1 and MHC class I molecules were observed, suggesting that aberrant presentation of antigenic peptides was involved in the pathogenesis of these diseases. In addition to these etiologic functions, the significance of the macrophage-activating activity of ERAP1 under specific pathological conditions should be elucidated.

In this context, we recently observed that secreted ERAP1 enhanced NO production from RAW264.7 cells in a substrate-specific manner, suggesting that ERAP1 is involved in the inflammatory response and blood pressure regulation. In summary, the results of this study suggest that IFN-β, TNF-α, and Ca2+/calmodulin play important roles in LPS/IFN-γ-induced secretion of ERAP1 from macrophages and contribute to their activation. Because cytokines and LPS are known to play important roles in the modulation of immunological/inflammatory/infecous processes by activating macrophages, our data provide new insights into the role of ERAP1 in the host defense mechanism. Further studies are required to elucidate the pathophysiological relevance of ERAP1 secretion from macrophages.

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Disclosures
The authors have no financial conflicts of interest.

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