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Ephrin-A1 expression induced by S100A8 is mediated by the toll-like receptor 4

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ABSTRACT

The deregulation of Eph/ephrin protein expression has been shown to lead to tumor development and progression. Both mRNA and protein expression analyses using clinical samples have demonstrated that ephrin-A1 is over-expressed in various cancers and positively correlates with a poor prognosis for cancer patients. The prognosis of cancer patients depends on metastasis to distant organs. We previously demonstrated that ADAM12 metalloproteinase cleaved ephrin-A1 and ADAM12-cleaved ephrin-A1 enhanced vascular permeability by degrading VE-cadherin and the EphA2 receptor at the plasma membrane. An increase of soluble ephrin-A1 levels in the serum facilitated tumor cell recruitment to the lungs, which resulted in lung metastasis. We also found that ephrin-A1 was overexpressed in 3LL tumors, a highly metastatic tumor, in mice and TNFα, an authentic positive regulator of ephrin-A1, was not elevated in the tumors, whereas S100A8 was. Moreover, S100A8 induced ephrin-A1 expression mediated by the toll-like receptor 4 (TLR4). S100A8 is known to be an endogenous ligand for TLR4 and its expression was shown to be increased in the lungs at the premetastatic phase. Thus, S100A8 and ephrin-A1 contribute to lung metastasis. Therefore, elucidating the regulation mechanism of ephrin-A1 overexpression is of importance and may lead to the development of therapeutic drugs against tumor growth and metastasis.

1. Introduction

Ephrin-A1 is a ligand of type-A Eph receptor tyrosine kinases and the Eph/ephrin system has been shown to play important roles in physiological events such as the nervous system, tissue patterning and cell adhesion through receptor–ligand interactions and intracellular signaling [1–3]. Therefore, dysfunctions of the Eph/ephrin system lead to various diseases. Previous studies using clinical samples showed that ephrin-A1 expression was up-regulated in colorectal cancer and hepatocellular carcinoma and positively correlated with a poor prognostic value [4,5]. We recently demonstrated that ephrin-A1 expression was higher in 3LL, a high metastatic tumor, than in LLC, a low metastatic tumor, in mice and the cleavage of ephrin-A1 by a disintegrin and metalloproteinase 12 (ADAM12) resulted in an increase of soluble ephrin-A1 in the serum. Soluble ephrin-A1 was previously shown to enhance lung vascular permeability and lung metastasis [6,7]. An increase of soluble ephrin-A1 in the blood stream has been associated with lung metastasis. The prognosis of cancer patients is known to depend on metastasis. Therefore, elucidating the mechanism for the up-regulation of ephrin-A1 expression is of importance and may lead to the development of therapeutic drugs for cancer and metastasis.

Ephrin-A1 was originally identified as an immediate early response gene to tumor necrosis factor α (TNFα), and was subsequently purified as a soluble factor in tumor conditioned medium [8,9]. Moreover, ephrin-A1 expression was recently shown to be up-regulated by S100A4, S100A8 and S100A9, mediated by receptor for advanced glycation end (RAGE) or CD147, also known as Emmprin [10–12]. However, the mechanism up-regulating the expression of ephrin-A1 in primary tumors remains unknown, and ligand–receptor specificity has not yet been investigated because previous reports did not perform loss-of-function experiments.

S100A8 and S100A9, also known as Myeloid-related protein-8 (MRP-8) and Myeloid-related protein-14 (MRP14), are calcium-binding proteins with EF-hand motif and are one of endogenous damage-associated molecular patterns (DAMPs), also referred to as alarmins. S100A8 and S100A9 were shown to be necessary for initiating the immune response to non-infectious inflammation [13], are abundantly expressed in myeloid lineage cells such as neutrophils, and are mostly released as heterodimers from

Abbreviations: Eph, erythropoietin-producing hepatocellular; LLC, Lewis lung carcinoma; MD-2, myeloid differentiation protein 2; SAA3, serum amyloid A3; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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dying cells or are secreted from immune cells activated by pathogen-associated molecular patterns (PAMPs) [14]. Previous studies demonstrated that S100A8 and S100A9 were increased in the serum due to rheumatoid arthritis and inflammatory bowel disease, and are known to be not only biomarkers in the inflammatory process for these diseases [15,16], but also play a pivotal role in amplifying inflammation. The complex of S100A8 and S100A9 was previously shown to bind to RAGE or CD147 and plays a crucial role in the inflammatory process. Although S100A8 and S100A9 are involved in amplifying the inflammatory process due to infection or inflammatory diseases, they also have been characterized as positive regulators of cancer development and tumor cell dissemination [17–19]. We and other groups have reported that S100A8 and S100A9 physically interacted with the TLR4/MD-2 complex, and this association was confirmed by Surface Plasmon Resonance (SPR) with K_D values of approximately 10 and 2 nM, respectively [19,20]. We also previously demonstrated that VEGF-A, TGF-β, and TNFα induced S100A8 expression in pre-metastatic lungs, and a TLR4-mediated S100A8-SAA3 paracrine system established a pre-metastatic niche in the lungs, leading to lung metastasis. The inhibition of proteins that establish a pre-metastatic niche in the lungs with neutralizing antibodies was shown to decrease the incidence of lung metastasis [19]. Consistent with our previous findings, S100A8 was shown to play an important role in lung metastasis.

As a step towards understanding the role of the overexpression of ephrin-A1 in primary tumors, we herein elucidated the mechanism that up-regulated the expression of ephrin-A1.

2. Materials and methods

2.1. Materials

Antibodies against ephrin-A1 (sc-911) and p65 (sc-372) were purchased from Santa Cruz (Dallus, TX). The antibody against actin was purchased from Millipore (Billerica, MA), that against phosphor p65 (#3033) antibody was purchased from Cell Signaling Technology (Danvers, MA), and that against mouse TLR/MD-2 antibody (13-9924) and the isotype control (13-4321) were purchased from eBioscience. FLAG agarose beads and mouse TNFα recombinant protein were purchased from WAKO laboratories (Osaka, Japan). LPS was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Plasmids

C-terminal FLAG-tagged full-length mouse S100A8 was subcloned into the *Kpn*I site of the pEBmulti-Hyg vector (WAKO).

2.3. Cell culture

Lewis lung carcinoma (LLC), Raw264.7, HEK293T and E0771 cells were cultivated in Dulbecco's modified Eagle's medium (WAKO) supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. HUVEC were obtained from Takara Bio (Shiga, Japan) and cultivated in human endothelial-SFM basal growth medium (Life Technologies, Carlsbad, CA) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin and low serum growth supplement (Life Technologies). 3LL tumor cells were cultivated as described previously [7]. F2 cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 mg/ml streptomycin and GlutaMax-I (Life Technologies).

2.4. Protein purification of mS100A8 protein

pEBmulti-mS100A8–FLAG was used for transfection into HEK293T cells. To isolate the S100A8 protein, transfected cells (5×10^8) were harvested and then lysed with 10 ml of extraction buffer (50 mM Tris–HCl, [pH 7.7], 150 mM NaCl, 1% Nonidet P40, and 0.5% sodium cholate). After brief centrifugation, the supernatant was passed through a filter unit with 0.45-μm pores and loaded onto 1 ml of anti-FLAG tag beads (WAKO). The column was washed with 5 ml of extraction buffer followed by washing with 10 ml of PBS. To remove the endotoxin, the beads were extensively washed with PBS/0.1% Triton X114 followed by PBS, which eliminated residual Triton X-114. S100A8–FLAG was eluted with 1 ml of FLAG peptide solution (0.1 mg/ml in PBS, Wako), and the effluent was concentrated with a centrifugal filter unit and dialyzed against PBS. The endotoxin levels of the purified mS100A8 protein were assessed by the Pyrochrome (Seikagaku, Tokyo, Japan) LAL method. Purified mS100A8–FLAG protein containing less than 0.01 EU/μg endotoxin was used for various assays.

2.5. Immunoblotting

Immunoblotting was performed as described previously [21]. To detect phosphorylated p65, cells were starved with DMEM(-) without FBS for 14 h followed by S100A8 or LPS stimulation. Protein concentrations were determined in the cell lysates using the BCA protein assay kit (Fisher Thermo Scientific, Waltham, MA). Twenty micrograms of the whole cell lysates were analyzed by SDS–PAGE and transferred onto a 0.45-μm pore-size polyvinylidene fluoride membrane (Millipore).

2.6. Immunofluorescence microscopy

Immunofluorescence staining was performed as described previously [21]. Images were obtained using a confocal laser scanning microscope (LSM710, Carl Zeiss, Oberkochen, Germany) and processed by Zen2011 imaging software.

2.7. Quantitative PCR

Total RNA was purified from tumor samples or cultured cells using ISOGENE II (Nippon Gene, Toyama, Japan). Complementary DNA was synthesized with ReverTra Ace reverse transcriptase (TOYOBO, Osaka, Japan) using 0.5 μg of total RNA with oligo dT and random primers. Quantitative PCR (qPCR) was performed using KAPA SYBR Green master mixture (Kapa Biosystems, Woburn, MA) with StepOne Plus (Life Technologies). Each mRNA level was normalized against β-actin. qPCR was performed using the following primer sets as shown in Supplementary Table 2.

2.8. RNA interference

Predesigned sequences were synthesized (mouse tlr4: TRCN0000065787) and ligated into the pLKO.1 lentivirus vector (Addgene plasmid 8453) to knockdown *tlr4* in LLC or E0771 tumor cells. LLC and E0771 cells infected with the lentivirus were selected with 2 μg/ml puromycin (InvivoGen, San Diego, CA).

2.9. Animal study

Animal studies were performed described previously [7].

2.10. Statistical analysis

Data are expressed as means ± S.D. Comparisons between two groups were performed with the two-tailed, paired Student's

t-test. In all experiments, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Ephrin-A1 expression in mouse primary tumors

Ephrin-A1 expression is up-regulated in some cancers such as colorectal cancer. We recently reported that ephrin-A1 expression was higher in 3LL, a high metastatic tumor, than in LLC, a low metastatic tumor, which were derived from Lewis lung carcinoma [7,22]. However, how the up-regulation of ephrin-A1 expression was regulated and what cells in these tumors expressed ephrin-A1 were unknown. Therefore, we investigated the expression profiles of ephrin-A1 in these tumors with CD31, an endothelial cell marker, or F4/80, a macrophage marker. We demonstrated that ephrin-A1 was expressed in tumor cells (Fig. 1A) and merged with F4/80, but not CD31, which suggested that ephrin-A1 was up-regulated in tumor cells and macrophages (Fig. 1B and C). Immunofluorescent staining with each isotype IgG control revealed the absence of staining in these tumors (Fig. 1D and E).

3.2. Up-regulation of ephrin-A1 expression by S100A8 in vitro

We analyzed TNFα mRNA levels in LLC and 3LL mouse tumors because stimulation with TNFα has been shown to induce ephrin-A1 expression in human umbilical vein endothelial cells (HUVECs) [23]. However, no significant difference was observed in TNFα mRNA levels between these tumors (Supplementary Fig. S1A). Whether ephrin-A1 expression was up-regulated by *in vitro* stimulation with TNFα was subsequently examined using cultured LLC and 3LL tumor cells. The TNFα treatment resulted in a significant increase in ephrin-A1 protein and mRNA levels in HUVECs, which was consistent with the findings of previous studies (Supplementary Fig. S1B and C). However, neither the protein nor mRNA levels of ephrin-A1 was elevated in LLC or 3LL tumor cells with the TNFα treatment even though both cell lines had sufficient the tumor-necrosis factor α receptors (TNFARs) to cause an elevation in *ccl2* mRNA expression (Supplementary Fig. S1D and E,

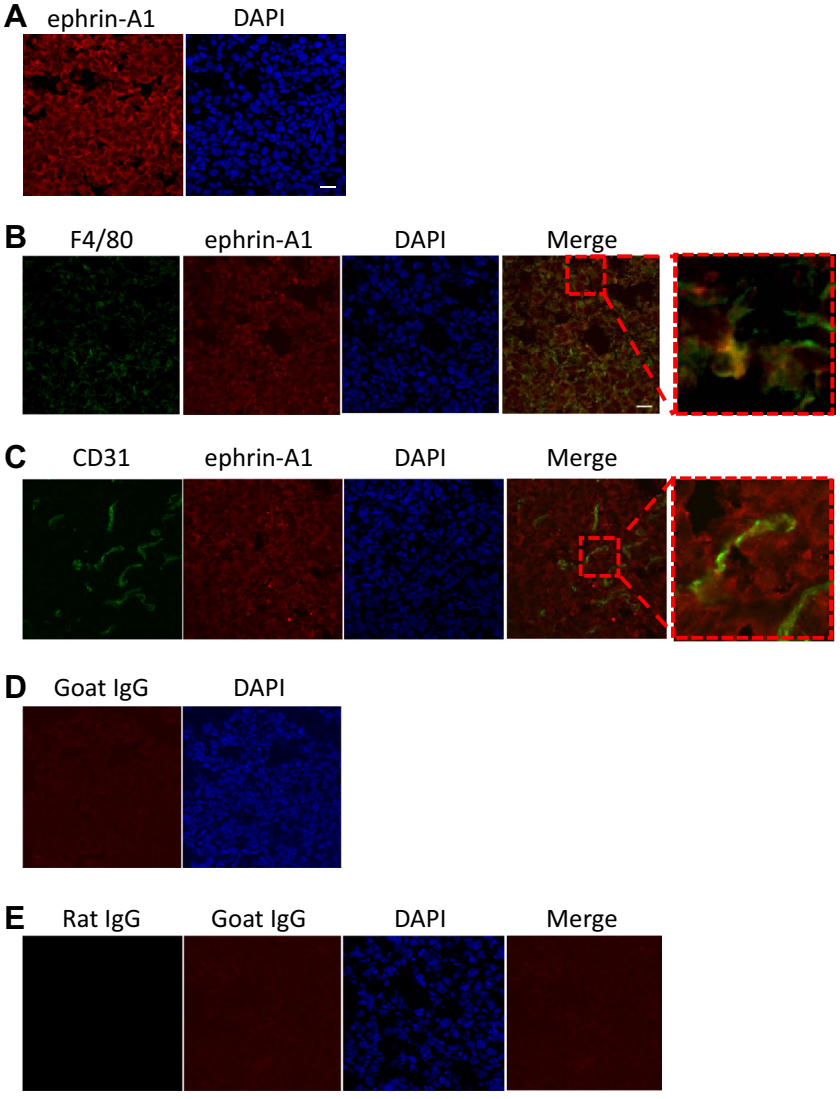

Fig. 1. Localization of ephrin-A1 in mouse primary tumors. (A–C) Localization of ephrin-A1 in primary tumors. Frozen sections obtained from mouse primary tumors were stained with an anti-ephrin-A1 antibody (red) with (B) F4/80 (green) or (C) with CD31 (green) (D–E) with the goat (red) or rat (green) isotype control. Scale Bar, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Supplementary Table S1). We also tested some mouse cell lines with the TNFα receptors including F2 angioma, E0771 mammary carcinoma, B16 melanoma, Hepa-1 hepatoma, Met-1 breast cancer, 3T3-L1 adipose, EL4 lymphoma, TC-1 myeloma, C22 clara, NIH3T3 fibroblast, MLE-15 lung epithelial and Raw264.7 macrophage cells to investigate if TNFα stimulation caused the up-regulation of ephrin-A1. Taken together with the results using LLC and 3LL tumor cells, most cell lines, except for E0771 cells, did not up-regulate the expression of ephrin-A1 in response to the TNFα stimulation (Supplementary Fig. S2).

Since S100A4, S100A8 and S100A9 were previously shown to induce ephrin-A1 expression mediated by RAGE and/or CD147 [10–12], we next examined their mRNA levels in LLC and 3LL tumors. S100A8 and S100A9, but not S100A4 mRNA levels were markedly higher in 3LL tumors than in LLC tumors (Fig. 2A). These results indicated that S100A4 was not involved in the up-regulation of ephrin-A1 expression during tumor growth or tumor progression in our mouse experimental model. We also measured *tlr4*, *md2*, *rage* and *cd147* mRNA levels in these tumors because S100A9 was previously shown to bind to RAGE, CD147, and TLR4/MD-2, and S100A8 to TLR4/MD-2 [12,19,24]. We demonstrated that *tlr4* mRNA levels were down-regulated in 3LL, whereas *rage* mRNA levels were up-regulated (Fig. 2B). Previous reports have shown that the S100A8/S100A9 complex binds to RAGE and CD147, whereas S100A8 does not [12,20]. We analyzed TLR4 in an attempt to simplify our focus on the up-regulation of ephrin-A1 expression because we previously demonstrated that SAA3 and S100A8 were endogenous ligands for TLR4 [19,24,25], and *saa3* mRNA levels were also higher in 3LL tumors than in LLC tumors (Supplementary Fig. S3). *Tlr4* mRNA levels in 3LL tumors appeared to be lower than those in LLC tumors (Fig. 2B). However, *in vitro* analysis using a flowcytometry revealed no significant difference in the surface protein expression levels of the TLR/MD-2 complex between LLC and 3LL tumor cells (Fig. 2C). We stimulated LLC and 3LL tumor cells with LPS, an authentic ligand for TLR4, to identify cells that respond to the NFκB transcription pathway because ephrin-A1 induction was previously shown to be driven by the NFκB transcription pathway [26,27]. HUVECs were used as a positive control. The results revealed a significant increase in ephrin-A1 protein expression (data not shown). The LPS treatment for 6 or 24 h induced ephrin-A1 protein expression in LLC and F2 cells (Fig. S4). Unexpectedly, 3LL tumor cells did not respond to the LPS stimulation, whereas LLC cells did. Because a 3LL tumor treated by Trypsin could not propagate *in vitro*, we established 3LL tumor cell

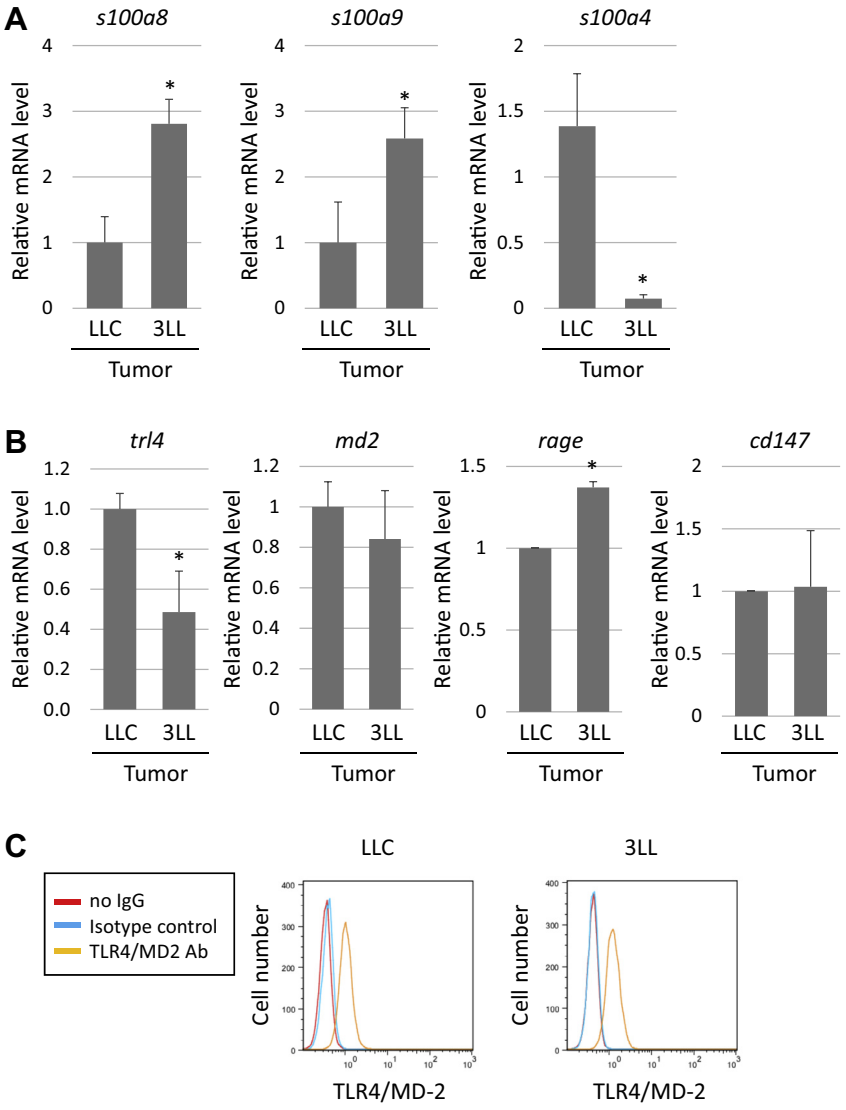

Fig. 2. mRNA expression profiles of S100As and their receptors in LLC and 3LL tumors. (A) qPCR analysis of S100As in LLC and 3LL tumors. ($n = 4$) $^*p < 0.05$ (B) qPCR analysis of *trl4*, *md2*, *rage* and *cd147* in LLC and 3LL tumors. ($n = 4$) $^*p < 0.05$ (C) A flowcytometric analysis of TLR4/MD-2 expression on the cell surface.

lines using a 3D culture gel. We previously used 3LL tumor cells for a lung metastasis assay *in vivo*. However, 3LL tumor cells were mostly defective in lung metastasis, which suggested different *in vivo* and *in vitro* functions [7], which may explain why 3LL tumor cells did not respond to the LPS stimulation. We next stimulated cells that responded to the TNFα or LPS stimulation with FLAG-tagged mouse S100A8 purified from HEK293T cells (mS100A8–FLAG) (Fig. 3A). The contamination of endotoxin was assessed by the LAL method before being used for assays and was subsequently not detected (less than 0.01 EU/ml/μg), which suggested that LPS contamination was negligible. Ephrin-A1 expression was up-regulated in E0771 and LLC cells, but not in F2 cells (Fig. 3B). We also examined whether stimulation with mS100A8–FLAG induced the phosphorylation of p65, a subunit of NFκB in E0771 cells, and showed that the level of p65 phosphorylation observed, although slight, was similar to that with the LPS stimulation (Fig. 3C).

3.3. Up-regulation of ephrin-A1 expression was mediated by TLR4

To investigate whether ephrin-A1 expression by S100A8 was mediated by TLR4, we abrogated TLR4 expression by the lentivirus shRNA vector system. Quantitative PCR analyses revealed a significant reduction in TLR4 expression (Fig. 4A). Moreover, the LPS stimulation did not induce an elevation in *tnfα* mRNA expression in cells in which TLR4 was abrogated (Fig. 4B and C). We stimulated E0771 cells lacking TLR4 expression with mS100A8–FLAG to examine whether ephrin-A1 expression was induced. Ephrin-A1 expression was induced by the S100A8 stimulation in E0771 cells expressing non-inhibitory shRNA, but not in TLR4-deficient E0771 cells, which suggested that ephrin-A1 expression upon stimulation with S100A8 is dependent on a TLR4 signal (Fig. 4D and E). These results demonstrated that the up-regulation of ephrin-A1 is mediated by the TLR4 signaling pathway *in vitro*.

4. Discussion

S100A8 is known to form a heterodimer with S100A9 and is secreted from neutrophils, activated monocytes and macrophages [28–30]. However, monomeric S100A8 and S100A9 were found in the skin and epithelium of a lung and influenced neutrophil migration and adhesion to the epithelium [31,32]. The functions of S100A8 and S100A9 have been characterized in many physiological events such as inflammation, but have not yet been fully elucidated. A high dose of S100A8/S100A9 was previously shown to induce apoptosis, whereas a low dose induced cell proliferation [33,34]. Thus, the functions of S100A8/S100A9 are controversial and have not been characterized in detail. To date, some S100 proteins have been identified that induce ephrin-A1 expression including S100A4, S100A8, S100A9 and the S100A8/A9 complex. However, previous studies did not perform loss-of-function experiments to determine ligand–receptor specificity. For example, although CD147 directly binds to S100A9, but not to S100A8, CD147 stimulated by S100A8 induced ephrin-A1 expression [12]. We consider this up-regulation to be independent of the CD147 signaling pathway. Treatment with S100A4 also induced the expression of ephrin-A1 in an NFκB-dependent manner, but receptor specificity was not examined in loss-of-function experiments. Thus, the detailed mechanism for the up-regulation of ephrin-A1 by S100 proteins has not yet been fully elucidated. We here demonstrated that S100A8, which had no direct interaction with RAGE or CD147, induced ephrin-A1 expression and this was mediated by the TLR4 signaling pathway. However, the downstream signal molecule of TLR4 needs to be clarified. Although we showed that macrophages in tumors expressed ephrin-A1, we did not observe ephrin-A1 induction by TNFα, LPS, or S100A8 using Raw264.7 or J774 cells. The LPS stimulation, but not S100A8 induced ephrin-A1 expression in F2 cells (data not shown). We speculate that the regulation mechanism for ephrin-A1 expression in macrophages and endothelial cells may differ from that in tumor cells or S100A4 and other proteins may induce ephrin-A1 expression in a different manner.

Previous studies demonstrated that a high dose more than 10 μg/ml of S100 proteins (A4, A8, A9) induced ephrin-A1 expression via the NFκB transcriptional pathway [10,12]. We showed that markedly lower concentrations of S100A8 induced ephrin-A1 expression in a TLR4-dependent manner. Although S100A8 concentrations of approximately 300–500 ng/ml have been measured in the serum of patients with diffuse cutaneous systemic sclerosis [35,36], which was similar to that of tumor-bearing mice, in hyperzincemia, the concentration of S100A8 was up to 1.4–6.5 mg/ml [13]. The concentration is high enough to induce ephrin-A1 expression via RAGE or CD147. Taken together, we suppose that RAGE- or CD147-dependent signaling contributes to an increase of ephrin-A1 expression in the acute phase in a time-dependent and site-specific manner or hyperzincemia, whereas TLR4-NFκB signaling by

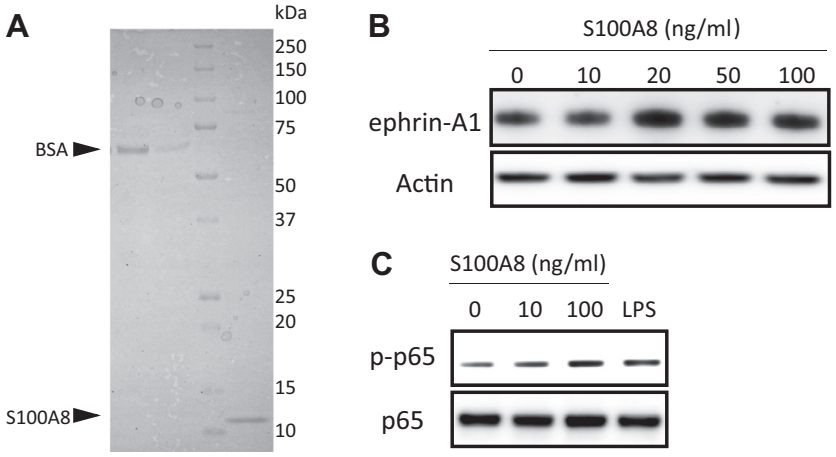

Fig. 3. Up-regulation of ephrin-A1 expression by S100A8. (A) SDS–PAGE of the mS100A8–FLAG protein purified from HEK293T cells stably expressing mS100A8–FLAG as described in the Materials and methods. Arrowheads show BSA (around 60 kDa) and mS100A8 (around 10 kDa). (B) Up-regulation of ephrin-A1 in E0771 cells. E0771 cells were stimulated with mS100A8–FLAG with the indicated concentrations for 9 h and were then analyzed by immunoblotting. (C) The effect of S100A8 stimulation on TLR4 signaling. E0771 cells were stimulated with S100A8 for 15 min and were then analyzed by immunoblotting.

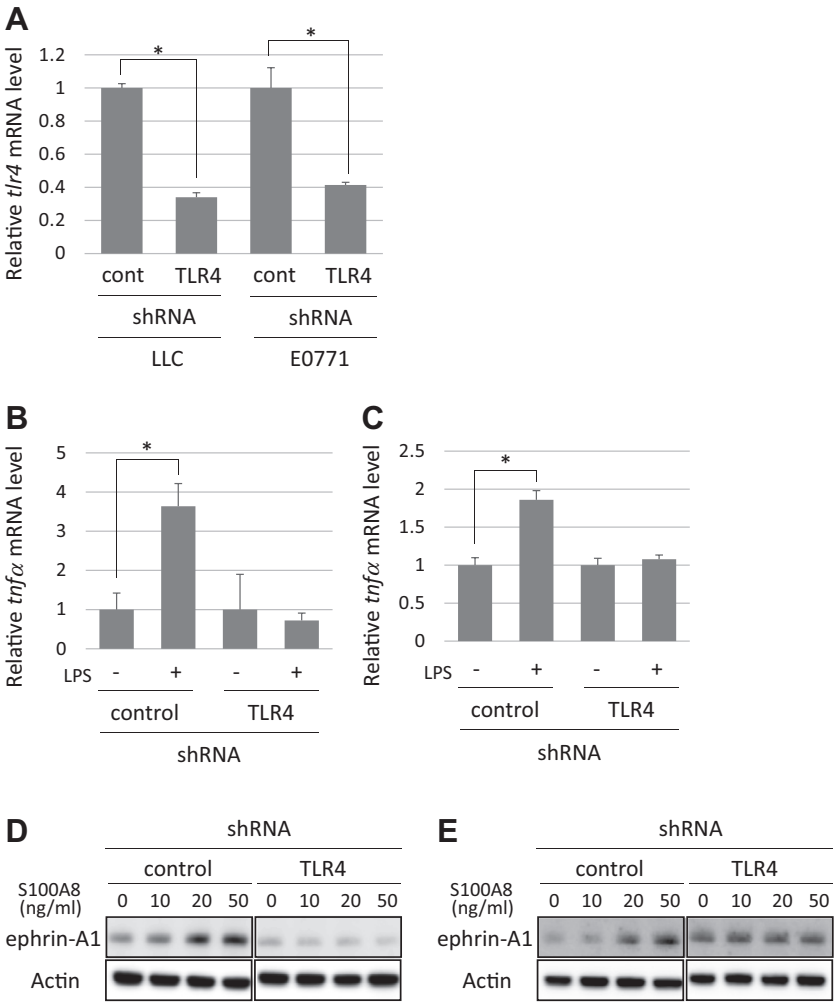

Fig. 4. TLR4-dependent up-regulation of ephrin-A1 expression by S100A8. (A) Knockdown of TLR4 by shRNA. LLC and E0771 cells were infected with lentivirus expressing TLR4 shRNA. Knockdown of TLR was confirmed by qPCR. $^{*}p < 0.05$ (B–C) TNFα induction by LPS in TLR4-deficient cells. Cells were stimulated with 1 μg/ml LPS for 90 min, and TNFα induction was tested by qPCR. $^{*}p < 0.05$ (D–E) Ephrin-A1 induction by S100A8 in TLR-deficient cells. LLC and E0771 cells in which TLR4 expression was abrogated were stimulated with S100A8 and analyzed by immunoblotting. $^{*}p < 0.05$ (B, D) LLC tumor cells. (C, E) 3LL tumor cells.

S100A8 induces ephrin-A1 expression in the chronic phase such as cancer. Moreover, S100 proteins may co-operatively regulate ephrin-A1 expression. The relationship between S100A8 and S100A9 or between TLR4, RAGE and CD147 for the up-regulation of ephrin-A1 expression requires further investigation.

In conclusion, we demonstrated that S100A8 mRNA levels were increased in primary tumors, and higher concentrations of S100A8 up-regulated ephrin-A1 expression mediated by TLR4. We previously reported that the expression of ADAM12 was higher in highly metastatic tumors than in low metastatic tumors [7]. We speculate that ADAM12 cleaves the ephrin-A1 induced by S100A8 in primary tumors, which enhances lung metastasis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.119.

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