Receptor for advanced glycation end products binds to phosphatidylserine and assists in the clearance of apoptotic cells

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Clearance of apoptotic cells is necessary for tissue development, homeostasis and resolution of inflammation. The uptake of apoptotic cells is initiated by an 'eat-me' signal, such as phosphatidylserine, on the cell surface and phagocytes recognize the signal by using specific receptors. In this study, we show that the soluble form of the receptor for advanced glycation end products (RAGE) binds to phosphatidylserine as well as to the apoptotic thymocytes. RAGE-deficient (*Rage*^{-/-}) alveolar macrophages showed impaired phagocytosis of apoptotic thymocytes and defective clearance of apoptotic neutrophils in *Rage*^{-/-} mice. Our results indicate that RAGE functions as a phosphatidylserine receptor and assists in the clearance of apoptotic cells.

Keywords: macrophage; phagocytosis; phosphatidylserine; apoptosis; RAGE

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INTRODUCTION

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell-surface

molecules (Neeper *et al*, 1992); it is expressed in diverse cell types including endothelium, T-lymphocytes and monocytes/macrophages (Brett *et al*, 1993). RAGE recognizes a variety of ligands, such as high-mobility group protein-1 (HMGB1), and the binding of RAGE to such ligands has been linked to many diseases including diabetes, atherosclerosis, neurodegeneration, inflammation and tumours (Ramasamy *et al*, 2009). RAGE is expressed in both a full-length membrane-bound form (mRAGE) and various soluble forms lacking the transmembrane domain (sRAGE). sRAGE is produced by the proteolytic cleavage of mRAGE and alternative mRNA splicing (Raucci *et al*, 2008; Hudson *et al*, 2008a) and is a competitive inhibitor of RAGE by acting as a decoy for RAGE ligands (Santilli *et al*, 2009).

Rapid removal of apoptotic cells by phagocytes is crucial for the maintenance of tolerance and prevents inflammation and auto-immune responses against intracellular antigens released from the dying cells (Savill & Fadok, 2000). The uptake of apoptotic cells is initiated by an 'eat-me' signal, such as phosphatidylserine, on the cell surface and phagocytes recognize the signal by using specific receptors (Fadok *et al*, 1992; Verhoven *et al*, 1995). Several classes of receptors have been implicated in the recognition of apoptotic cells, possibly by means of direct binding to the exposed phosphatidylserine, including brain-specific angiogenesis inhibitor 1 (BAI1), T-cell immunoglobulin and mucin domains-containing protein 4 (Tim4) and stabilin-2 (Kobayashi *et al*, 2007; Miyanishi *et al*, 2007; Park *et al*, 2007, 2008), or indirect binding through bridging molecules (milk fat globule-EGF factor 8 protein (MFG-E8), growth arrest-specific 6 (Gas6); Scott *et al*, 2001; Hanayama *et al*, 2004). The engagement of the phosphatidylserine receptors initiates signalling events within the phagocytes that lead to activation of the small GTPase Rac and the subsequent cytoskeletal reorganization of the phagocyte membrane, to allow corpse internalization (Albert *et al*, 2000). Deficiencies of the receptors that participate in the recognition of phosphatidylserine on the surface of apoptotic cells

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Fig 1 | sRAGE binds to apoptotic cells and phosphatidylserine. (A) JC-1 dye detects mitochondrial membrane potential and red fluorescence decreases in apoptotic cells (black dots). sRAGE specifically bound to apoptotic cells (black), compared with a non-apoptotic population (grey). Data shown are representative of three experiments. (B) sRAGE bound to phosphatidylserine blotted on PIP Strip. Data shown are representative of two experiments. (C) SPR assays. A serial concentration of sRAGE (0, 4.32, 13.9, 117 and 350 μ g/ml) was flowed over immobilized PS or PC liposomes on the Biacore sensor chip. Response to PC was used as a reference (left). The response curve of PS/sRAGE binding was obtained by subtracting the curve of PS from that of PC, as described in the Methods section (right). Data shown are representative of two experiments. BSA, bovine serum albumin; PC, phosphatidylcholine; PS, phosphatidylserine; RU, response units; SPR, surface-plasmon resonance; sRAGE, soluble receptor for advanced glycation end products.

lead to a failure to maintain self-tolerance and the development of autoimmunity (Cohen *et al*, 2002; Asano *et al*, 2004).

Although RAGE is a member of the pattern-recognition receptor family (Xie *et al*, 2008), whether it participates in the phagocytosis and clearance of apoptotic cells remains unknown. In this study, we present evidence that RAGE binds to phosphatidylserine and modulates apoptotic cell phagocytosis. Furthermore, we confirm the role of RAGE in apoptotic cell clearance in lipopolysaccharide (LPS)-induced acute lung injury in mice.

RESULTS AND DISCUSSION

sRAGE binds to apoptotic thymocytes and phosphatidylserine

To determine whether RAGE identifies its functional ligands on apoptotic cells, we prepared thymocytes with a proportion of apoptotic cells by dexamethasone treatment and evaluated the binding capacity of RAGE to the apoptotic thymocytes by using

Alexa Fluor 660 dye-labelled sRAGE and JC-1 dye (Petit *et al*, 1995). We found that sRAGE specifically bound to an apoptotic population of thymocytes (Fig 1A, black portion), indicating that RAGE binds to a structure highly expressed on the surface of apoptotic thymocytes. Advanced glycation end product, one of the main RAGE ligands, was not present on apoptotic thymocytes (supplementary Fig S2 online). Exposure of phosphatidylserine on the outer-membrane leaflet is an important indicator of the presence of apoptotic compared with live cells (Fadok *et al*, 1992); therefore, we tested whether RAGE can bind to phosphatidylserine. By using a protein-lipid overlay assay that used hydrophobic membranes pre-spotted with a concentration gradient of eight different lipids found in cell membranes (PIP Strip), we found that sRAGE specifically bound to phosphatidylserine in a concentration-dependent manner, except for a slight binding to phosphatidylglycerol (Fig 1B). To determine the binding affinity of sRAGE to phosphatidylserine,

Fig 2 | RAGE and phosphatidylserine bind to each other on the cell surface. (A) Images of the donor (NBD-PS, green) on apoptotic thymocytes, the acceptor (sRAGE, red), the bleached area and the FRET spots are shown. FRET efficiency was significantly increased by bleaching the fluorescence of the acceptor (sRAGE), demonstrating the direct binding between PS and RAGE (right graph). Data represent average values with s.e.m. Student's *t*-test was used to analyse the comparisons between two groups, and *P* values < 0.05 were considered significant. (B) A representative confocal microscopic image of a macrophage phagocytosing a PS liposome. Membrane-associated full-length RAGE expression on macrophages was concentrated in a pseudopod that was formed (arrow) towards a PS liposome (arrowhead). Phagocytosed NBD-PS was seen in the cytoplasm of the macrophage. DAPI, 4,6-diamidino-2-phenylindole; DIC, differential interference contrast; FRET, fluorescence resonance energy transfer; mRAGE, membrane receptor for advanced glycation end products; PS, phosphatidylserine; RAGE, receptor for advanced glycation end products; sRAGE, soluble receptor for advanced glycation end products.

we used a surface-plasmon resonance analysis (Biacore) using phosphatidylserine or phosphatidylcholine (as a reference) liposomes immobilized on the sensor chip and serial dilutions of sRAGE as soluble analytes. The sensorgrams showed an increase in response units that was reflective of phosphatidylserine binding, and the binding response was concentration dependent, with a K_D of 0.563 μ M (Fig 1C). These findings indicate that sRAGE specifically binds to phosphatidylserine.

To investigate whether sRAGE can interact with phosphatidylserine on apoptotic cells, apoptotic thymocytes were incubated with NBD-phosphatidylserine (1,2-dioleoyl-sn-glycero-3-phospho-L-serine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)) and Chromeo 546-labelled sRAGE. The distribution of phosphatidylserine and sRAGE on the cell surface was determined by confocal imaging, and the interaction between sRAGE and NBD-phosphatidylserine was measured by fluorescence resonance energy transfer (FRET analysis). As shown in Fig 2A, both phosphatidylserine and sRAGE were localized to the surface of apoptotic thymocytes. FRET spots, indicating interactions between sRAGE and phosphatidylserine, appeared on the cell surface, with the acceptor (Chromeo 546-sRAGE, red fluorescence) being photobleached. By contrast, fewer FRET spots were observed on the cell surface of the same thymocytes that were not photobleached (Fig 2A). This indicates that RAGE and phosphatidylserine colocalize on the cell membrane.

Confocal images of alveolar macrophages cocultured with NBD-phosphatidylserine liposomes showed that mRAGE expression was concentrated in a pseudopod that formed towards a phosphatidylserine liposome (Fig 2B). Both FRET analysis and confocal images demonstrated that phosphatidylserine expressed on apoptotic cells binds to full-length mRAGE located in macrophages, and that this phosphatidylserine-RAGE binding might have a role in apoptosis recognition and phagocytosis.

RAGE deficiency or sRAGE impairs phagocytosis

As RAGE can bind to phosphatidylserine, we sought to determine the function of RAGE in the phagocytosis of apoptotic cells, examining the phagocytic capacity of both wild-type and *Rage*^{-/-} alveolar macrophages (Fig 3A). Alveolar macrophages were cocultured with apoptotic thymocytes from wild-type mice. The phagocytic index of wild-type alveolar macrophages at 2 h was significantly higher than that of *Rage*^{-/-} macrophages ($P = 0.013$; Fig 3B), suggesting that alveolar macrophages can recognize and phagocytose apoptotic cells through the activity of RAGE on their cell surface. Because sRAGE functions as an endogenous competitive inhibitor of ligand engagement by cell-surface RAGE (Santilli et al, 2009), we then examined the effect of sRAGE on macrophage phagocytosis. sRAGE administration significantly impaired the phagocytosis of apoptotic thymocytes by wild-type alveolar macrophages, which confirms that RAGE contributes to

Fig 3 | *Rage*^{-/-} alveolar macrophages impair the phagocytosis of apoptotic thymocytes *in vitro* and show reduced Rac1 activation in response to phosphatidylserine liposomes. (A) Representative microscopic images showing the phagocytosis of wild-type and *Rage*^{-/-} alveolar macrophages with or without sRAGE (3 µg/ml) treatment. Arrowheads indicate phagocytized thymocyte fragments. (B) Wild-type alveolar macrophages or *Rage*^{-/-} alveolar macrophages incubated with apoptotic thymocytes with or without sRAGE. The number of apoptotic cells per macrophage uptake was determined as the phagocytic index. Phagocytic indexes were significantly decreased in sRAGE-treated and RAGE-deficient macrophages. (C) PS, and not PC, activated Rac1 in wild-type macrophages. PS-induced Rac1 activation was not seen in RAGE-deficient macrophages. sRAGE significantly attenuated PS-induced Rac1 activation. White bars represent wild type; black bars represent *Rage*^{-/-}. Data represent average values with s.e.m. The data were compared using analysis of variance with the Bonferroni adjustment, and *P* values <0.05 were considered significant. AM, alveolar macrophage; PC, phosphatidylcholine; PS, phosphatidylserine; RAGE, receptor for advanced glycation end products; *Rage*^{-/-}, RAGE deficient; sRAGE, soluble receptor for advanced glycation end products; WT, wild type.

the phagocytosis of apoptotic cells. Interestingly, sRAGE administration also induced a significant phagocytosis defect in *Rage*^{-/-} alveolar macrophages; it is possible that sRAGE counteracts not only RAGE-mediated phagocytosis, but also other phosphatidylserine receptor-mediated phagocytosis, such as that mediated by BAI1 and Tim4. These results suggest that RAGE is one of the phosphatidylserine receptors that recognize apoptotic cells.

PS–RAGE binding is crucial for Rac1 activation

Intracellular RAGE signalling pathways involve Rho GTPases (Hudson *et al*, 2008b) and Rac1, activation of one of Rho GTPases is essential in the phosphatidylserine-recognition system that is involved in apoptotic cell removal (Albert *et al*, 2000). We therefore prepared phosphatidylserine liposomes to mimic apoptotic cells and tested whether phosphatidylserine–RAGE binding could alter the level of activated Rac1 during uptake by alveolar macrophages. Wild-type alveolar macrophages showed greater phosphatidylserine liposome-induced Rac1 activation; by contrast, phosphatidylserine liposomes did not activate Rac1 in *Rage*^{-/-} alveolar macrophages. Phosphatidylcholine liposomes were tested as a negative control, and did not activate Rac1 in either the wild-type or *Rage*^{-/-} alveolar macrophages (Fig 3C). These data demonstrate that inducible Rac1 activation is due to phosphatidylserine recognition through RAGE in alveolar macrophages. The level of Rac1 remained at near-basal levels in both wild-type and *Rage*^{-/-} macrophages when phosphatidylserine

liposomes were supplied with sRAGE (Fig 3C). sRAGE competed with RAGE for phosphatidylserine binding and functioned as a decoy that abrogated Rac1 activation, supporting the assertion that phosphatidylserine–RAGE binding is a prerequisite for the induction of Rac1 activation in alveolar macrophages.

Defective clearance of apoptotic cells in *Rage*^{-/-} mice

We next tested whether RAGE participates in apoptotic cell clearance *in vivo*. As RAGE is constitutively expressed at high levels in the lung (Brett *et al*, 1993), we induced lung injury in *Rage*^{-/-} and wild-type mice by an intranasal insufflation of LPS (20 µg per mouse in 100 µl of PBS). Intranasal instillation of LPS induces extensive neutrophil migration within the airspace. Once neutrophils extravasate, they undergo programmed cell death (Watson *et al*, 1996) and are then removed from the airspace by phagocytosis of alveolar macrophages (Cox *et al*, 1995). This active programme of resolution starts in the first few hours after an inflammatory response begins (Serhan & Savill, 2005). We performed a bronchoalveolar lavage (BAL) at 0 (baseline), 2, 4, 6 and 24 h after LPS administration and examined the apoptotic cells recovered from the BAL fluid. More apoptotic neutrophils were found in *Rage*^{-/-} mice at 6 and 24 h after LPS administration than in wild-type mice (Fig 4A). Further, we examined the phagocytosis of both wild-type and *Rage*^{-/-} alveolar macrophages (Fig 4B, right). *Rage*^{-/-} alveolar macrophages showed significantly decreased phagocytosis of apoptotic neutrophils at 6 and

Fig 4 | *Rage*^{-/-} alveolar macrophages impair apoptotic cell clearance *in vivo*. (A) Cells recovered from BAL fluid after LPS exposure were stained with FITC-conjugated annexin V and phycoerythrin-conjugated mouse Ly-6G (GR-1) antibody. Data show average counts of apoptotic neutrophils (GR-1⁺/annexin V⁺) in wild-type mice and *Rage*^{-/-} mice ($n=3-5$). (B) Alveolar macrophage phagocytosis of apoptotic neutrophils in LPS-induced BAL was determined as the AM phagocytic index ($n=6-9$). The image indicates an AM with an ingested apoptotic neutrophil. (C) Changes in total cell counts in BAL fluid (left) and neutrophils (right) after LPS exposure in wild-type mice and *Rage*^{-/-} mice, at each time point ($n=5-9$). (D) Changes in KC (left) and MIP-2 (right) levels in BAL fluid during the 24 h after LPS exposure ($n=6-9$). White bars represent wild type; black bars represent *Rage*^{-/-}. Data represent average values with s.e.m. throughout. The data were compared using analysis of variance with the Bonferroni adjustment except neutrophil counts, KC and MIP-2. Neutrophil counts, KC and MIP-2 were analysed using non-parametric analysis of variance (Kruskal-Wallis test) with Dunn's Multiple Comparison Test. P values <0.05 were considered significant. AM, alveolar macrophage; BAL, bronchoalveolar lavage; FITC, fluorescein isothiocyanate; KC, keratinocyte-derived chemokine; LPS, lipopolysaccharide; MIP-2, macrophage inflammatory protein 2; *Rage*^{-/-}, receptor for advanced glycation end products deficient; WT, wild type.

24 h, compared with wild type (Fig 4B, left). Next, we checked whether this impaired clearance of apoptotic neutrophils would affect the accumulation of inflammatory cells within the airspace. We found that LPS administration resulted in a rapid increase of inflammatory cells in BAL fluid at 2, 4, 6 and 24 h in mice. The total number of cells in BAL fluid recovered from the *Rage*^{-/-} mice was substantially greater than that recovered from the wild-type mice at 2, 4, 6 and 24 h, and there was a statistically significant difference at 6 h between the two groups ($P=0.02$; Fig 4C, left). The increased number of inflammatory cells was largely attributable to the increase in neutrophils (Fig 4C, right). We also checked keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP-2), which are the key

cytokines associated with neutrophil recruitment (Martin, 2002). The KC and MIP-2 levels in BAL fluid did not significantly differ between the two types of mice after LPS administration (Fig 4D). Taken together, these data show that deletion of RAGE in macrophages impairs the clearance of apoptotic cells, thereby leading to increased inflammatory cell accumulation within the airspace in the early phase of LPS-induced acute lung injury.

In LPS-induced lung injury, HMGB1 has a key role in the late phase of the injury (Wang *et al*, 1999). Liu *et al* (2008) reported that HMGB1 interacts with phosphatidylserine and diminishes the phagocytosis of apoptotic cells. We confirmed the binding of HMGB1 and phosphatidylserine by protein-lipid overlay assay (supplementary Fig S3A online) and surface-plasmon resonance

Fig 5 | Summary of a possible RAGE- and sRAGE-signalling pathway in phagocytosis that involves phosphatidylserine recognition. (A) We propose that RAGE is a PS receptor that functions similarly to stabilin-2, BAI1 and Tim-4. (B) By binding to PS, sRAGE might counteract RAGE and other PS receptors, such as BAI1 and stabilin-2. BAI1, brain-specific angiogenesis inhibitor-1; mDia-1, mammalian Diaphanous-1; PS, phosphatidylserine; RAGE, receptor for advanced glycation end products; Tim-4, T-cell immunoglobulin and mucin domains-containing protein 4; sRAGE, soluble receptor for advanced glycation end products.

analysis (Biacore; supplementary Fig S3B online). However, HMGB1 also bound to other lipids—namely, phosphatidylglycerol and phosphatidic acid—and its binding affinity to phosphatidylserine was lower than that of sRAGE ($K_D = 3.08 \mu\text{M}$ in HMGB1, $K_D = 0.563 \mu\text{M}$ in sRAGE), according to the Biacore analysis. Nevertheless, there remains the possibility of a multiprotein complex composed of sRAGE and HMGB1 that binds to phosphatidylserine *in vivo*; further work is needed to clarify this.

Apoptotic cell clearance is important for tissue development (Savill & Fadok, 2000), and inefficient phagocytosis of apoptotic cells could lead to the development of autoimmune disease (Zitvogel et al, 2010). However, *Rage*^{−/−} mice that were kept in a sterile environment, were viable and did not show any overt phenotypes. The lack of both RAGE and sRAGE might keep the RAGE-null animals in a comparative balance in which no phenotype is expressed.

The data presented in this study provide new insights into the process of apoptotic cell clearance by RAGE. First, RAGE might

function as one of the phosphatidylserine receptors that recognize and initiate apoptotic cell clearance (Fig 5A). Second, our data raise an important point regarding an sRAGE/mRAGE balance in overall apoptosis recognition, because sRAGE might counteract the phosphatidylserine engagement of cell-surface RAGE and other receptors, such as BAI1, Tim4 or stabilin-2, during phagocytosis (Fig 5B). sRAGE is produced by alternative splicing of RAGE mRNA or by proteolytic cleavage of RAGE from the cell surface in humans, and sRAGE has the same ligand-binding specificity, resulting in competition with RAGE for ligand binding. As sRAGE functions as a competitor for mRAGE binding to phosphatidylserine, the balance of sRAGE and mRAGE could modify macrophage phagocytotic activity, which might be an important component of pathophysiological situations in which apoptosis recognition has a key role.

The present finding that RAGE is a phosphatidylserine receptor reveals a new role for RAGE in apoptosis-induced pathophysiological conditions and identifies a potential new target for the treatment of the relevant human diseases.

METHODS

Recombinant sRAGE protein and *Rage*^{−/−} mice were used for this study.

Identification of sRAGE by flow cytometry. Alexa Fluor 660 dye-labeled sRAGE or bovine serum albumin was added to dexamethasone-treated thymocytes. Apoptotic cells were identified using a MitoProbe JC-1 assay kit (Molecular Probes). Flow cytometry was performed on a BD FACSCanto II (BD Biosciences).

PIP strip assay. PIP strips on which the indicated phospholipids had been spotted, were purchased from Echelon Bioscience, and dot-blot experiments were carried out according to the manufacturer's protocol.

Surface-plasmon resonance analysis (Biacore). The binding affinity of sRAGE to phosphatidylserine was analysed using a Biacore X100 (Biacore AB) in a single-cycle affinity model. K_D was calculated using k_a and k_d values by a trivalent analyte model.

FRET analysis. FRET analysis was performed as described previously (Liu et al, 2008).

Confocal laser scanning microscopy. Alveolar macrophages were grown on coverslips and then preincubated with $5 \mu\text{M}$ NBD-phosphatidylserine liposome for 2 h, after which mRAGE was detected by indirect immunofluorescence using a rabbit polyclonal RAGE antibody (Abcam) with Alexa Fluor 647-conjugated goat rabbit IgG (Molecular Probes).

Phagocytosis of apoptotic thymocytes. Phagocytosis was assayed by adding apoptotic thymocytes (2.5×10^6 cells/ml) with or without sRAGE ($3 \mu\text{g/ml}$). Phagocytosis was determined as a phagocytic index under microscopy, as described previously (Morimoto et al, 2006). Each condition was tested in duplicate, and the samples were analysed blindly and independently by two researchers (M.H. and K.M.).

Rac1 activity assays. Rac1 activity was measured using an ELISA-based Rac1 Activation Assay Biochem Kit (G-LISA, Cytoskeleton).

LPS-induced lung injury. LPS from *Escherichia coli* serotype 055:B5 was obtained from Sigma-Aldrich, and the induction of lung injury was performed as described previously (Yamada et al, 2004).

Detailed methods are described in the supplementary information online.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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