Human eosinophils express RAGE, produce RAGE ligands, exhibit PKC-delta phosphorylation and enhanced viability in response to the RAGE ligand, S100B

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Abstract
This study tested the hypothesis that human eosinophils produce ligands for the receptor for advanced glycation end-products (RAGE), express RAGE and exhibit RAGE-mediated responses. In examining our microarray data, we identified the presence of RAGE and RAGE ligand (S100A4, S100A6, S100A8, S100A9, S100A11, S100P, HMGB1) transcripts. Expression of eosinophil RAGE mRNA was also compared with a known positive control and further assessed via bioinformatics and sequence analysis of RAGE cDNA. Positive and negative controls were used to identify RAGE, S100A8 and S100A9 protein in human primary eosinophils. Immunoblot assessment of eosinophils treated with cytokines (IL-5 or granulocyte macrophage colony-stimulating factor) indicated an up-regulation of S100A8 and S100A9 production, whereas co-treatment of eosinophils with a RAGE ligand and cytokines displayed a down-regulation in the levels of RAGE. Analysis of eosinophil-conditioned media revealed that eosinophils are capable of releasing RAGE, S100A8 and S100A9. To test the eosinophil response to RAGE activation, the most well-characterized RAGE ligand, S100B, was examined. Treatment of eosinophils with S100B resulted in RAGE-mediated PKC-delta phosphorylation, a 3-fold dose-dependent increase in cell survival and an increase in the level of cellular RAGE. Combined, these studies reveal eosinophil expression of RAGE, soluble RAGE and RAGE ligands may be pivotal to the functions of eosinophils in various human diseases involving RAGE and S100 ligands.

Keywords: CD11b, GM-CSF, IL-5, S100A8, S100A9

Introduction
Eosinophils are bilobed granulocytic cells predominantly known to be recruited in response to chronic inflammatory diseases (1). Of relevance to the present report, the expression, activation and release of the receptor for advanced glycation end-products (RAGE) contribute to disease-associated inflammation (2, 3). A subset of RAGE ligands, the S100 proteins, is prominently expressed in many human cancers with indicated functions in tumor progression (4, 5). The S100B homodimer and S100A8/S100A9 heterodimer are expressed in tumors known to exhibit eosinophilia such as melanoma and colon cancer (4, 6). The S100B protein is also a CD8+ T-cell inflammatory cytokine involved in central nervous system pathologies (7, 8). Eosinophils have been reported to produce and respond to neuromediators, associate with peripheral and enteric nerves, and induce neurotoxic activity (9, 10). The eosinophil is also an established effector cell in the pathobiology of lung and gastrointestinal inflammation (10–13). Originally characterized as cystic fibrosis antigens, the S100A8/S100A9 proteins are increasingly implicated in chronic airway and intestinal disease (14, 15). Together, this information suggests that S100 proteins may play a role in the eosinophil response. However, the intracellular and extracellular mechanisms that enable eosinophils to participate in various human afflictions are not fully known.

RAGE is a multiligand receptor known to interact with structures formed via non-enzymatic glycation of proteins (advanced glycation end-products), amyloid-beta, S100/calgranulins, high-mobility group box-1 (HMGB1) and Mac-1 (CD11b/CD18, αβ integrin, complement receptor 3) (16). In certain cell types, such as monocytes and lung epithelial
cells, RAGE can exist as a full-length isofrom on the cell membrane and as soluble isofroms as a result of alternative splicing of pre-mRNA or proteolytic cleavage (17–19). The development and recruitment of macrophages are indicated in RAGE-mediated disease progression, resulting in RAGE ligand-induced macrophage activation and the production of proinflammatory cytokines (16, 20). Studies have also indicated that macrophage RAGE functions as a ligand for phosphatidylserine in the clearance of apoptotic cells (21, 22). Of interest, increased expression of RAGE ligands is associated with eosinophilia (23), suggesting that, like macrophages, eosinophils may also possess RAGE-mediated functions. In examining our previously published microarray of human primary blood eosinophils (24), RAGE and RAGE ligand mRNA was detected. This preliminary insight suggests that the RAGE system may play a pivotal role in the eosinophilic response. To date, the study of RAGE or RAGE ligands in eosinophils has not been extensively explored.

Eosinophils are myeloid cells formed in response to regulatory signals provided by the cytokines IL-3, IL-5 and granulocyte macrophage colony-stimulating factor (GM-CSF) (25). Eosinophils are also capable of diverse activities involving tissue remodeling/repair, immunological responses and neurological interactions that are not well understood (9, 10). Interestingly, RAGE ligands are implicated in these same functional responses (16). For example, S100B at nanomolar concentrations has a neurotrophic effect, encouraging repair/regeneration responses. However, at higher concentrations, S100B functions as a neurotoxin and activates microglia (26). Excessive activation of macrophages, tissue damage and necrosis are linked to the activities of RAGE ligands (27) and likewise the recruitment of eosinophils (25, 28). These findings support a potential role of eosinophils in RAGE-mediated wound repair, inflammation and neurological function. Therefore, the aim of the present work was to determine if eosinophils express RAGE and RAGE ligands and if eosinophils are responsive to a known inflammation-associated RAGE ligand, namely S100B.

Methods

Isolation and culture of peripheral blood and bronchoalveolar lavage eosinophils

Peripheral blood and bronchoalveolar lavage (BAL) fluid was obtained from human allergic rhinitis and allergic asthmatic patients under informed consent. The study was approved by the University of Wisconsin–Madison Center for Health Sciences Human Subjects Committee. Relevant patient information was made available in accordance with institutional review board protocols. Blood eosinophils were purified from heparinized blood that was diluted with Hank’s buffered saline solution (HBSS; Mediatech, Manassas, VA, USA) without Ca2+ and centrifuged for 20 min at 700 × g over 1.090 g ml−1 Percoll. A granulocyte fraction was obtained after removal of the plasma, mononuclear cell band and Percoll. Granulocytes were then subjected to RBC lysis via hypotonic shock, washed with 4°C HBSS supplemented with 2% newborn calf serum (Life Technologies, Grand Island, NY, USA) and incubated 40 min with magnetic beads coated with anti-CD16, anti-CD14 and anti-CD3 (Miltenyi Biotechnology, Auburn, CA, USA) prior to negative selection with an AutoMACS separator (Miltenyi Biotechnology). The recovered mixture (>97% purity and >98% viability) was evaluated by Giemsa’s-based Diff-Quik stain (Baxter Scientific Products, McGaw Park, IL, USA) and trypan blue exclusion, respectively. Blood eosinophils were cultured in RPMI 1640 (Mediatech) containing 0.001 or 0.1% human serum albumin (HSA; Irvine Scientific, Santa Ana, CA, USA). Airway eosinophils were purified by centrifugation of BAL cells through a Percoll bilayer (1.085/1.100 g ml−1). Eosinophils were recovered at the interface between the Percoll layers and resuspended in room temperature HBSS supplemented with 2% newborn calf serum. Airway eosinophil purity (>99%) and viability (>97%) were identified as described in the blood eosinophil isolation.

Isolation and culture of peripheral blood monocytes

Human blood-derived monocytes were purified as previously described (29). In brief, heparinized peripheral blood was obtained from volunteer donors and enriched for peripheral blood mononuclear cells via centrifugation through a Percoll monolayer. Platelets were removed by three washes in HBSS (Mediatech) with 2% heat-inactivated bovine serum. Monocytes were enriched with RosetteSep monocyte enrichment cocktail and a standard Ficoll centrifugation. The monocytes were collected at the Ficoll/plasma interface and dispensed (2 × 106 cells/ml) into 12-well plates (Costar, Corning, NY, USA) with RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin/streptomycin (Mediatech) and 2 mM L-glutamine (Mediatech) (RPMI 10% complete media). After 2 h in culture at 37°C, cells were rinsed with warm HBSS (Mediatech) to remove non-adherent lymphocytes and resuspended in RPMI 10% complete media for an additional 24 h at 37°C prior to lysis and processing.

Cell lines

The human HEK-293 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The human H358 non-small-cell lung cancer (NSCLC) and DU145 prostate carcinoma cell lines were kindly provided by Dr Paul Harari and Dr Wade Bushman (University of Wisconsin, Madison, WI, USA), respectively. The DU145 prostate carcinoma and HEK-293 cells were cultured in DMEM (Mediatech) supplemented with 10% Cosmic Calf Serum (Hyclone) and 100 U/ml penicillin/streptomycin (Mediatech). The H358 NSCLC cells were cultured in RPMI 10% complete media.

Reagents for cell stimulation

Cells, when indicated, were stimulated with GM-CSF (R&D Systems, Minneapolis, MN, USA), IL-5 (R&D Systems) or N-formyl-methionyl-leucyl-phenylalanine (fMLF; Sigma Chemical Co., St Louis, MO, USA). The S100B ligand (Calbiochem/EMD Biochemicals, Inc., Gibbstown, NJ, USA) was used to activate RAGE given its commercial availability, its known receptor interactions (30) and its reported functions in certain cancers (4) and central nervous system pathologies (7) that have been indicated to recruit eosinophils (6, 9).
Analysis of RAGE mRNA expression

Eosinophils (8–10 x 10^6 cells) were lysed by repeated passage with a 27.5-gauge needle in Qiagen RNaseasy Mini Kit Buffer (Qiagen, Valencia, CA, USA). Lysates were subjected to a freeze/thaw (−80°C, 15 min or O/N) to ensure adequate cell lysis and enriched total RNA (0.8–2 µg) was isolated in association with the manufacturer's protocol (Qiagen RNaseasy Mini Kit). Synthesis of cDNA was performed with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) and 0.5–1 µg RNA. PCR was performed in a 10 µl reaction solution (HotStarTag Master Mix Kit; Qiagen) containing cDNA corresponding to 50 ng total RNA and 0.5 µM primer (each for reverse and forward primers). The cycling conditions were 95°C for 5 min, followed by 40 cycles, each consisting of denaturation at 94°C for 30 s, annealing at 52°C for 3.5 min and extension at 60°C for 1.5 min and a final extension at 72°C for 10 min. The PCR products were analyzed on 1 or 1.5% agarose gels and visualized by staining with ethidium bromide. Bands were subsequently excised and purified using QIAquick Gel Extraction Kit (Qiagen). The purified PCR product sequences were fluorescently labeled with BigDye Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA) and 0.5–1 pmol dye. Amplification of RAGE cDNA was performed using primer pairs targeting untranslated regions (UTR) and internal sequences of the gene similar to previous study by Hudson et al. (17). Electrophoresis of PCR product and DNA ladder (HyLadder 10 kb; Denville Scientific Inc., Metuchen, NJ, USA) were performed on a 1% agarose gel. Primers utilized are as follows: 5’ primer (RAGE5'UTR1, 5'-AGGAACGAGGATGAGCAGC-3'); 3’ primer (RAGE3'UTR1, 5'-GTCTGAGGCCAGAACGATTC-3'); 5’ primer (RAGEexon8, 5'-GCCCTGCTGCTACCTCCTGGAG-3'); 5’ primer (RAGEexon7, 5’-GCCAGAAGGTGGAGCAGGA-3'); 3’ primer (RAGEexon4, 5’-AGGCAGAATCTACAATTCCTGGC-3').

Bioinformatics

DNA sequence determination was performed with Genescan software at the University of Wisconsin Biotechnology Center. Sequences corresponding to RAGE were identified in GenBank using the BLAST Human Sequences tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence data were viewed and annotated using the BioEdit program (31) and aligned to RAGE mRNA (NM_001136.3, CDS 25-1239) using Clustalw (http://www.ebi.ac.uk/clustalw/) and the nucleotide BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Immunoblotting

Cells were solubilized with lysis buffer (1% SDS, 10 mM dithiothreitol, 0.5 mM Na3VO4, 1 mM EDTA, 10% glycerol, 10 mM Tris, pH 8.0), sonicated, and boiled (5 min). For analysis of PKC-δ phosphorylation/activation, the cell lysates were additionally subjected to a freeze/thaw cycle (−80°C, 15 min) and a centrifugation (10 min, 15 800 x g) step prior to isolating the soluble fraction. Concentrated cell lysis buffer (10×) was added to S100A8/S100A9-conditioned media (1:10 dilution) and boiled (5 min). Soluble RAGE-conditioned media was added to ice-cold acetone (1.3, −20°C, 60 min) and the protein precipitate was pelleted via centrifugation (4°C, 10 min, 15 800 x g), suspended in lysis buffer (1×), sonicated and boiled (5 min). Protein concentrations were determined by Micro-BCA protein assay reagents (Thermo Scientific Pierce, Rockford, IL, USA) and loaded onto a 10, 12.5 or 15% SDS–PAGE gel. Proteins were transferred onto 0.45 µm Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA), incubated with antibodies raised against human RAGE (R&D Systems goat polyclonal AF1145, 1:500), actin (BD Biosciences, San Jose, CA, USA; 612656, 1:5000), phospho-PKC-δ (Cell Signaling Technology, Inc., Danvers, MA, USA; 9374, 1:500). Total PKC-δ (Cell Signaling Technology, Inc.; 2058, 1:500), S100A8 or S100A9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-8112, sc-8114, 1:200). Blots were washed and subsequently incubated with horseradish peroxide (HRP)- conjugated secondary antibodies. The immunoblots that were re-probed for total PKC-δ were stripped with One Minute® Western blot Stripping Buffer (GM Biosciences, Inc, Rockville, MD, USA), incubated with secondary antibodies and examined for negative chemiluminescence. Bound secondary antibody was visualized following incubation of the membrane with Super Signal West chemiluminescent HRP substrate (Thermo Scientific Pierce) and an Epichem II darkroom equipped with a 12-bit cooled CCD camera. Luminescence was quantified and evaluated using ImageJ software (National Institutes of Health).

Immunofluorescence

Eosinophils were adhered to coverslips via cytopsin, fixed in 4% paraformaldehyde, treated with 0.1% Triton X-100 and blocked with PBS containing 1% bovine serum albumin (Sigma) and 1 µg ml⁻¹ normal rabbit IgG (Millipore; 12–370). Cells were immunostained with polyclonal goat antibody raised against human RAGE (Santa Cruz sc-8230; 1:50), human S100A8 (Santa Cruz sc-8112; 1:50), S100A9 (Santa Cruz sc-8114; 1:50) or isotype control. The isotype control and primary antibodies were reacted with rabbit anti-goat Alexa Fluor 488 antibodies (Invitrogen, Carlsbad, CA, USA; A-11708, 1:2000). The nucleic acid stain DAPI was added to identify the bi-lobed nucleus of eosinophils (Invitrogen; 1:10 000). Coverslips were mounted onto slides with Fluoro-Gel w/Tris Buffer (Electron Microscopy Sciences, Hatfield, PA, USA) prior to visualization using a Nikon epi-fluorescence microscope.

Survival analysis, CD11b expression and activation

Purified blood eosinophils (2 x 10⁶/ml) were cultured atop 150 µl 0.5% w/v agarose-coated 48-well tissue culture plates (Sarstedt, Newton, NC, USA). Cells were treated with increasing concentrations of S100B (Calbiochem/EMD Biochemicals, Inc.) and compared with cells treated with buffer control or GM-CSF (R&D Systems). Eosinophil viability was determined by assessing the exclusion of propidium iodide (PI; 3 µg ml⁻¹) staining/fluorescence. The expression and activation state of CD11b were, respectively, identified by first incubating with αM/CD11b (MAB1699; R&D Systems) or
CBRM1/5 (301402; Biolegend, San Diego, CA, USA) antibodies followed by reaction with a secondary 488 Alexa Fluor-conjugated donkey anti-mouse antibody (A-21202; Invitrogen) and by comparison with an isotype control. All analyses were performed using a FACSscan flow cytometer (Becton-Dickinson, Bedford, MA, USA).

**Statistical analyses**

Measurements of CD11b expression and activation were assessed among groups using mixed-effects analysis of variance models with a fixed-effect covariate per group and a random-effect covariate to account for within-patient correlation of measurements. All additional analyses were performed using a paired Student’s t-test. A two-sided P value of <0.05 was regarded as statistically significant.

**Results**

**Detection of mRNA for RAGE and RAGE ligands in human primary blood eosinophils**

RAGE has multiple reported ligands and has been found to be expressed in cells of a myeloid lineage (16). In addition, a previous study has suggested the expression of RAGE in human primary eosinophils (32). To elaborate upon these results, we analyzed our previous microarray data of human primary blood eosinophils stimulated with control buffer, 100 pM IL-5 or 100 pM GM-CSF (4 h) (24). Transcripts identified as present are summarized in Fig. 1 and compared with known cytokine responsive transcripts, CD69 and proviral integration site for Moloney murine leukemia virus 1 (PIM-1). These data revealed the presence of message for RAGE and RAGE ligands (S100A4, S100A6, S100A8, S100A9, S100A11, S100P, HMGB1) in human primary blood eosinophils.

To confirm RAGE mRNA expression, human primary blood eosinophils were obtained from three different donors and compared with DU-145 prostate carcinoma cells, an established positive RAGE control (33). Cells were lysed and mRNA was isolated as detailed under Methods. Primers specific to the UTR and internal exons were used to amplify cDNA in the presence or absence of reverse transcriptase. Electrophoresis of PCR product revealed a migrating band comparable to RAGE mRNA (coding sequence 25-1239, NM_001136.3). A representative image of three separate analyses displays the expression of RAGE in DU-145 prostate carcinoma cells and eosinophils in the presence of reverse transcriptase (Fig. 2), verifying the presence of cDNA and not genomic DNA.

**Sequence and bioinformatics analysis of human primary blood eosinophil RAGE**

Bioinformatic analysis was performed for additional confirmation of eosinophil RAGE expression. Sequences of cDNA obtained from eosinophils of two separate donors were subjected to a BLAST search (Fig. 2), revealing identity with only one gene in the human genome, the advanced glycosylation end-product-specific receptor, Gene ID: 177. Alignment of the full sequences with RAGE mRNA (coding sequence 25-1239, NM_001136.3) revealed identity of >97%, supporting our data (Fig. 1), indicating eosinophil RAGE transcript expression.

**Identification of RAGE protein expression in human primary blood eosinophils**

To determine if eosinophils express RAGE at the protein level, the H358 NSCLC cell line and DU-145 prostate carcinoma cell lines were used as controls due to their respective lack and presence of RAGE protein (33–35). Because each cell type differs in their cellular concentrations of actin, the controls are restricted to identifying, and not comparatively quantifying, RAGE protein expression in eosinophils. In Fig. 3A, a representative immunoblot identifying RAGE in eosinophils is displayed. Repeat experiments involving three different donors were assessed for RAGE levels using densitometry and normalization to actin controls, and these data, which support the idea that RAGE is expressed by human eosinophils, are presented in Fig. 3B. In addition, as shown in Fig. 4, immunofluorescence microscopy with an
RPMI were cultured for 1 h with control buffer, 10^6 cells/ml human primary blood eosinophils suspended in 0.1% HSA RPMI induces eosinophil RAGE expression, 2
expression (36). In examining if the RAGE ligand, S100B, interaction has been reported to amplify RAGE
expression in response to S100B treatment compared with
conditioned media were obtained from these cultures and loaded
onto 12.5% SDS–PAGE gels, transblotted and immunostained
for RAGE. In Fig. 6A, a representative immunoblot from four
different eosinophil donors is shown wherein RAGE immunoreactivity is identified in both the cell lysates and in the conditioned media. Densitometry analyses were performed and these data indicated enhanced soluble RAGE expression with increased culture time and GM-CSF treatment (Fig. 6B).

S100A8 and S100A9 production by human primary blood eosinophils

S100A8 is a common inflammatory protein that has been reported to function as a dimer with S100A9 in cells of a myeloid lineage (38). As shown in Fig. 1, mRNA expression levels of S100A8 appear to be responsive to GM-CSF and IL-5 treatment in human primary blood eosinophils. To test whether S100A8 or S100A9 protein production are responsive to these cytokines, human primary blood eosinophils in 0.1% HSA RPMI (2 × 10^6 cells/ml) were cultured for 1 h with control buffer, 100 pM GM-CSF or 100 pM IL-5. Samples were separated on a 15% SDS–PAGE gel and compared with human blood monocytes, which is a cell type known to express S100A8 and S100A9 (38), as well as HEK-293 cells, which serves as a negative control. As shown in Fig. 7A–C, eosinophils from nine different donors expressed an 11 and a 14 kDa protein comparable to monocyte control samples. Addition of GM-CSF or IL-5 to the media significantly enhanced the expression of the bands depicting eosinophilic S100A8 and S100A9 at 1 h. The presence of these proteins was further evaluated via immunofluorescence microscopy and found to be expressed throughout the eosinophil (Fig. 7B and C). In addition, analysis of BAL eosinophils revealed the presence of S100A8 and S100A9 in lung eosinophils (Fig. 7D).

S100A8 and S100A9 release by human primary blood and BAL eosinophils

Increased levels of S100A8 and S100A9 have been indicated to occur in mucosal inflammation (39). To assess if eosinophils can release these proteins, human primary blood and BAL eosinophils (5 × 10^6 cells/ml) were cultured for 24 h in 0.1% HSA RPMI in the presence of control buffer, 100 pM GM-CSF or 100 pM IL-5. Cell lysates and conditioned media were obtained from these cultures and loaded onto 15% SDS–PAGE gels, transblotted and immunostained for S100A8 or S100A9. In Fig. 8A and B, representative immunoblots from a total of seven different eosinophil donors are shown wherein S100A8 and S100A9 immunoreactivity are identified in both the cell lysates and the conditioned media. Densitometry analyses of immunoblot data were

RAGE protein expression by human primary blood eosinophils in response to S100B treatment. RAGE ligand–receptor interaction has been reported to amplify RAGE expression (36). In examining if the RAGE ligand, S100B, induces eosinophil RAGE expression, 2 × 10^6 cells/ml human primary blood eosinophils suspended in 0.1% HSA RPMI were cultured for 1 h with control buffer, 10 μg/ml S100B, 100 pM GM-CSF or 100 pM IL-5. In Fig. 5A, a representative immunoblot for RAGE is displayed. These data suggest that S100B can induce RAGE expression in human primary blood eosinophils. Repeat experiments involving five different donors were assessed for RAGE levels using densitometry and normalizing to actin controls. These results reveal a significant increase (P = 0.005) in eosinophil RAGE expression in response to S100B treatment compared with the buffer control (Fig. 5B). Treatment with GM-CSF or IL-5 alone or in combination with S100B did not appear to significantly affect cellular RAGE expression.

Soluble RAGE protein production by human primary blood eosinophils. The most common variants of RAGE include the full-length form, the C-truncated (soluble RAGE) form and the N-truncated isoforms (36). RAGE isoforms have been proposed to occur via alternative splicing of the pre-mRNA (17) or in response to proteolytic cleavage by metallopro-

**Fig. 2.** Reverse transcriptase (RT)–PCR detection of human primary blood eosinophil RAGE by direct observation of ethidium-stained PCR products. cDNA was generated in the presence or absence of RT and amplified with primers specific to the UTR of RAGE as detailed in Methods. Bands displayed correlate in size with RAGE mRNA (coding sequence 25–1239, NM_001136.3). A representative image is displayed from three separate experiments involving the DU145 cell line and three different eosinophil (EOS) donors.

additional RAGE polyclonal antibody (see Methods) revealed receptor expression throughout the cytoplasm, thereby supporting our immunoblot assessment (Fig. 3A and B) of RAGE protein expression in human primary blood eosinophils. Analysis of BAL eosinophils also revealed the presence of RAGE in lung eosinophils (Fig. 3C).

Eosinophils express RAGE and RAGE ligands

Eosinophils express RAGE and RAGE ligands, such as A Disintegrin And Metalloprotease 10 (ADAM10) and matrix metalloproteinase 9 (MMP-9) (37). In examining our previous microarray data of human primary blood eosinophils, transcripts for both ADAM10 and MMP-9 were identified as being present in human eosinophils (24). To examine if soluble RAGE is produced by human primary blood eosinophils, these cells (2 × 10^6 cells/ml) were incubated for 2 or 4 h in 0.001% HSA RPMI and in the presence of control buffer or 100 pM GM-CSF. Cell lysates and conditioned media were obtained from these cultures and loaded onto 12.5% SDS–PAGE gels, transblotted and immunostained for RAGE. In Fig. 6A, a representative immunoblot from four different eosinophil donors is shown wherein RAGE immunoreactivity is identified in both the cell lysates and in the conditioned media. Densitometry analyses were performed and these data indicated enhanced soluble RAGE expression with increased culture time and GM-CSF treatment (Fig. 6B).
performed where trends suggest increased S100A9 production in response to treatment with GM-CSF or IL-5 after 24-h culture. In addition, analysis of BAL eosinophil-conditioned media also indicated the release of S100A8 and S100A9 by these cells (Fig. 8E).

**S100B induced eosinophil PKC-δ phosphorylation**
RAGE-mediated activation of PKC-δ (as assessed by its phosphorylation state) has been previously shown to occur in neurons (40). To test whether eosinophils can also rapidly (within 5 min) respond to RAGE ligands, eosinophils were examined for PKC-δ phosphorylation in response to fMLF, a known activator of PKC-δ (41), or in response to S100B treatment. As illustrated in Fig. 9, S100B induced a significant increase (>50%) in the level of eosinophil PKC-δ phosphorylation (using either actin or total PKC-δ loading controls), and this observation is consistent with role for PKC-δ in mediating S100B responses in human eosinophils.

**Effects of S100B treatment on CD11b expression/activation, cell size and viability**
The S100 proteins have been found to enhance the expression of the adhesion receptor CD11b and to increase cell survival in monocytes and neurons, respectively (42). In eosinophils, these responses are regulated by the activation of PKC-δ (43), a serine and threonine kinase we observed to be activated by S100B (Fig. 9). To examine S100B-induced CD11b expression, eosinophils from six different donors were cultured in 0.1% HSA RPMI and treated with buffer control, 1 pM GM-CSF or 10 µg ml⁻¹ S100B for 24 h and assessed for CD11b expression and activation. As shown in Fig. 10A, where all donors were analyzed in aggregate, CD11b surface detection was not significantly affected by S100B treatment, although two donors did exhibit an up-regulation in the immunodetection of the active CD11b compared with the vehicle control. In addition, as presented in Fig. 10B, treatment of eosinophils with GM-CSF led to the expected and significantly augmented CD11b surface detection and activation compared with the vehicle control. Of note, addition of S100B to GM-CSF-treated eosinophil cultures, as shown in Fig. 10C, appeared to alter the response of several donors when compared with GM-CSF treatment alone (Fig. 10B). When directly assessing potential differences in eosinophil CD11b responses between GM-CSF and GM-CSF plus S100B treatments (Fig. 10D), we observed distinct but differential changes in the detection of CD11b surface expression and activation, indicating
donor-dependent variations in response to GM-CSF versus GM-CSF plus S100B. Furthermore, in Fig. 10E–G, data are shown illustrating that cell size and PI staining are reduced by treatment with GM-CSF or GM-CSF plus S100B compared with the control. These data indicate that eosinophils cultured above agarose and treated with GM-CSF plus S100B are more morphologically uniform in cell size and more viable when compared with other cell treatment groups.

S100B-induced alterations in eosinophil survival

Cytokines pivotal to eosinophilic function, such as IL-5 and GM-CSF, are known to enhance eosinophil survival (25). To further test whether eosinophil survival is affected by S100B administration, eosinophils from four different donors were cultured for 1 h in 0.1% HSA RPMI in the presence of buffer control, 100 pM GM-CSF, 100 pM IL-5 or 10 μg ml^{-1} S100B. Cell lysates (50 μg) of protein were loaded onto a 10% SDS–PAGE gel and examined for RAGE expression by immunoblotting (A). Data were quantified with ImageJ software and expressed as RAGE mean band densitometry relative to actin controls, ±SEM, N = 5 (B).
Fig. 6. Detection of eosinophil soluble RAGE (sRAGE) protein. Human primary blood eosinophils \((2 \times 10^6/\text{ml})\) were cultured in 0.001% HSA RPMI in the presence of buffer control or 100 pM GM-CSF. Total eosinophil cell lysates from \(1 \times 10^6\) cells or the total corresponding conditioned media were loaded onto a 12.5% SDS–PAGE gel and immunoblotted as detailed under Methods. Immunoblots detecting soluble and cellular RAGE from a representative eosinophil donor are displayed (A). Immunoblot data from multiple patients were quantified with ImageJ software and expressed as RAGE mean band densitometry relative to actin controls, ±SEM, \(N = 4\) (B).

Fig. 7. Immunoblot detection of cellular levels of eosinophil S100A8 and S100A9. Human primary blood eosinophils (EOS) were cultured for 1 h in 0.1% HSA RPMI (\(2 \times 10^6/\text{ml}\)) in the presence of buffer control, 100 pM GM-CSF or 100 pM IL-5. Cell lysates (50 ìg of protein) were loaded onto a 15% SDS–PAGE gel and immunoblotted for S100A8 (A and B) and S100A9 (A and C); protein expression was compared with positive (human peripheral blood monocytes (Mono)) or negative (HEK-293) controls via immunoblot (A) and densitometry analysis with ImageJ software (B and C). A total of 11 samples of protein were examined from nine different donors where two blots were probed for both proteins. Data are expressed as S100A8 or S100A9 mean band densitometry relative to actin, ±SEM: treatment versus control; \(* P < 0.05\), \(N = 6\) (S100A8), \(N = 5\) (S100A9). BAL EOS S100A8 and S100A9 cellular expression were also assessed in comparison to blood EOS from the same or different donor, \(N = 3\) (D).
Fig. 8. Immunoblot detection of extracellular eosinophil S100A8 and S100A9. Human primary blood eosinophils (5 × 10^6/ml) were cultured in 0.1% HSA RPMI in the presence of buffer control, 100 pM GM-CSF or 100 pM IL-5 for 24 h. Total eosinophil cell lysates from 5 × 10^5 cells and the total corresponding conditioned media were loaded onto a 15% SDS–PAGE gel. Representative immunoblots for S100A8 and S100A9 are, respectively, displayed in (A) and (B). Data were also quantified with ImageJ software and expressed as S100 mean band densitometry relative to actin controls (C and D), 6 SEM, N = 3 (S100A8), N = 4 (S100A9). BAL eosinophil S100A8 and S100A9 extracellular expression were also assessed by immunoblot (E).

Fig. 9. S100B-induced phosphorylation of PKC-δ in human eosinophils. Human primary blood eosinophils (2 × 10^6/ml) were cultured in 0.1% HSA RPMI in the presence of buffer control, fMLF (100 nM) or S100B (10 μg ml^-1) for 5 min. Cell lysates (100 μg of protein) were loaded onto a 10% SDS–PAGE gel and probed for PKC-δ phosphorylation. Assessment of PKC-δ phosphorylation was ascertained by immunoblot (A and B) and quantified by ImageJ densitometry analysis (C and D). Data are expressed as PKC-δ mean band densitometry relative to actin, 6 SEM: treatment versus control; *P < 0.05, N = 5 (C) or PKC-δ mean band densitometry relative to total PKC-δ (T-PKC-δ), 6 SEM: treatment versus control *P < 0.05, N = 3 (D).
neuronal apoptosis and the activation of astrocytes and microglia (26).

Discussion
Despite the discovery of eosinophils over a century ago, insight into the biochemical functions of these granulocytic, myeloid cells in various human diseases has remained elusive (44). Of note, the presence of RAGE ligands and the expression, activation and release of RAGE have been identified in chronic inflammatory disorders associated with eosinophils (1–7, 9, 11, 12, 14). To explore the potential presence and function of RAGE in eosinophils, we examined our microarray data (Fig. 1) and confirmed the presence of

Fig. 10. Continued
eosinophil RAGE transcripts by examining mRNA from cells obtained from three different donors (Fig. 2). Bioinformatic analyses on the cDNA sequences obtained from two separate donors also revealed identity with the RAGE gene. We identified eosinophil RAGE protein in both blood and lung eosinophils via immunoblot analysis (Fig. 3). In addition, immunofluorescent detection of RAGE in eosinophils revealed a distributed pattern (intracellular and plasma membrane associated; see Fig. 4). Such a pattern may represent multicomartment trafficking and activation of the receptor and would be consistent with possible RAGE ligation and internalization via one of the many eosinophil RAGE ligands (Fig. 1) (45). The distributed pattern is also noted in the immunofluorescent detection of S100A8 and S100A9 (Fig. 4), which are two proteins that have been found to intracellularly co-localize with RAGE (46). In addition, we determined that S100B treatment can induce cellular RAGE production (Fig. 5), whereas cytokines tend to reduce the detection of cellular RAGE but increase the detection of soluble RAGE (Figs 5 and 6), suggesting that cytokines may activate cell signals involved proteolytic mechanisms. Interestingly, this form of soluble RAGE has previously been reported in monocytes (18) and may be increased in the lung following cytokine exposure, which would be consistent with our observation, that cellular RAGE levels appear to be lower in lung versus blood eosinophils (Fig. 3). It is noteworthy that the RAGE gene has been mapped to human chromosome 6p21.3 in the human major histocompatibility

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**Fig. 10.** S100B induced CD11b, size and viability responses after 24 h in culture. Detection of CD11b expression and activation (A–D). Human primary blood eosinophils (2 × 10^5/ml) were cultured in 0.1% HSA RPMI atop 0.5% w/v agarose and assessed via flow cytometry (see Methods) for CD11b expression and activation in response to buffer control, GM-CSF (1 pM) or S100B (10 μg ml⁻¹) after 24 h of treatment. Cells were negatively selected for PI stain and positively selected for CD11b 488 Alexa Fluor fluorescence. The CD11b data are displayed as the average 488 geometric mean fluorescence (GMF) of eosinophils from six donors, ±SEM. Analyses were performed comparing control versus S100B treatment (A), control versus GM-CSF treatment (B), control versus GM-CSF + S100B (C) and GM-CSF versus GM-CSF + S100B (D). Detection of size and viability changes (E–G). Representative density plot images display eosinophil size (E) and viability (F) in response to buffer control, GM-CSF (1 pM) or S100B (10 μg ml⁻¹) after 24 h of treatment. Cell gates were established on the GM-CSF + S100B sample and copied to all other samples. Increased cell size is identified by forward scatter (FSC) and marked by a solid line (E). Increased cell death is identified by PI stain and marked by a dashed line (F). Percentages marked in Panels E and F were tabulated and charted (G), ±SEM. *P < 0.0001 versus control, †P = 0.04 versus control, ‡P = 0.007 versus control. Data displayed (A–G) associate smaller cell size and increased viability with CD11b expression and activation.
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complex (MHC) class III locus near the junction with MHC class II (47, 48) and that altered expression of the classical and non-classical MHC genes has been linked to various diseases, such as diabetes, asthma, rheumatoid arthritis and cancer (49). Together, these data suggest that the expression of RAGE, soluble RAGE and RAGE ligands by eosinophils may play an important role in the progression and persistence of multiple disorders, including chronic inflammatory diseases.

In our microarray investigations, the S100A8 and S100A9 transcripts were also identified as present (Fig. 1). The S100A8 and S100A9 proteins are RAGE ligands (50) that have been reported to be involved in the differentiation of myeloid cells (50, 51). In examining eosinophil S100A8 and S100A9 expressions, we found that without stimulation, eosinophils expressed low and comparable levels of both S100A8 and S100A9 proteins (Fig. 7) throughout the cytoplasm and potentially at the cell surface (Fig. 4). Interestingly, up-regulation of the S100A8 and S100A9 proteins has been indicated to occur in a RAGE-dependent manner (5). RAGE and the cytokines, GM-CSF and IL-5, reportedly induce many of the same intracellular signals [p38 (52, 53), PKC-δ (40, 54), JAK2 (55, 56) STAT5 (56, 57)], suggesting that GM-CSF and IL-5 may activate RAGE or indirectly induce cell signals in common with RAGE for S100A8 and S100A9 production. In response to a 1-h treatment with 100 pM GM-CSF or IL-5, we found an increase in the expression of both proteins in human primary eosinophils (Fig. 7). S100A8 and S100A9 are structural proteins known to promote tubulin polymerization and regulate the migratory capacity of phagocytic cells (58). These proteins may therefore play a role in the process of eosinophil priming where enhanced cell signaling in response to chemokines has been found to occur subsequent to ‘priming’ eosinophils for 1 h with either IL-5 or GM-CSF (59). We found the presence of S100A8 and S100A9 transcripts in eosinophils after 4 h of incubation, but only the mRNA levels of S100A8 increased in response to the addition of GM-CSF or IL-5 (Fig. 1). This

Fig. 11. S100B induced eosinophil survival after 48 h in culture. Human primary blood eosinophils were cultured in 0.1% HSA RPMI atop 0.5% w/v agarose in the presence of buffer control, GM-CSF (100 pM) or S100B (0.3, 1, 3, 10, 30 µg ml⁻¹) for 48 h. The percentage of viable cells was determined by exclusion of PI (3 µg ml⁻¹) via flow cytometric analysis as visualized (A) and charted (B). Cell gates were established on the viable cells in the GM-CSF-treated sample, which were >70% viable. Cell percentages are averaged, ±SEM: *P = 0.02 versus control, †P = 0.007 versus control, N = 4.
result may indicate that S100A9 transcription is comparatively rapid and is sufficient to stabilize S100A8, a mechanistic function of S100A9 that has been previously explored (58, 60) and/or that control of S100A9 protein levels can also occur post-transcriptionally. After 24 h of incubation, S100A8 and S100A9 proteins were identified in eosinophil-conditioned media, indicating that these proteins are released by eosinophils (Fig. 8). Elevated human S100A8/ S100A9 serum levels are found in patient studies of type 1 and 2 diabetes mellitus (61, 62), cystic fibrosis (63), cardiac sarcoidosis (64), acute coronary syndrome (65), Kawasaki disease (66), inflammatory bowel disease (15), acute pancreatitis (67), psoriasis (68), juvenile idiopathic arthritis (69), rheumatoid arthritis (70), acute allograft rejection (71, 72), malaria (73) and cancers of the colon (74) and ovary (75). Possible eosinophil degranulation associated with S100A8 and S100A9 release, by IL-5- or GM-CSF-activated eosinophils, may aid in our understanding of chronic mucosal inflammatory diseases, such as cystic fibrosis and inflammatory bowel disease, which are characterized by the presence of these S100 proteins as well as the eosinophilic granule proteins (14, 15, 76, 77).

Aside from S100A8 and S100A9, other RAGE ligands such as S100A4, S100A6, S100A10, S100A11 and S100P were also identified as present on the microarray (Fig. 1), whereas S100A1, S100A2, S100A3, S100A5, S100A7, S100A12, S100B and S100G were reported as absent (data not shown). RAGE is indicated to interact with the S100 proteins identified as present in the microarray with the exception of S100A10 (26, 50). This latter protein (S100A10/annexin II ligand/calpain I) is predominantly known to co-localize with annexin A2, leading to the organization of cytoskeletal and membrane proteins (78). S100A11 (calgazzarin) has similar interactions via annexin A1 and reported extracellular functions in regulating keratinocyte growth and differentiation (79, 80). S100A4 (metastasin), S100A6 (calcyclin) and S100P are produced by various cell types and like most S100 proteins are found up-regulated in human cancer (4). These attributes of S100 proteins may offer insight into the interactions of eosinophils with S100B and additional proteins known to enhance eosinophilic granule proteins (14, 15, 76, 77).

Eosinophil cell survival, changes in cell shape and CD11b detection have been previously found to be regulated by PKC-δ (43, 84, 85), a cell signal molecule we show to be phosphorylated in response to micromolar levels of S100B (Fig. 9). Previous research has indicated that nanomolar concentrations of S100B can induce neurotrophic effects and repair/regeneration responses, whereas micromolar levels induce neurotoxicity and microglia activation (26). In our study, we found that micromolar levels of S100B enhanced eosinophil viability (Fig. 11), which may, in part, account for the association of eosinophils in certain types of central nervous system pathologies.

In examining CD11b responses in eosinophils, we found that S100B can alter CD11b expression and activation in a subset of individuals, particularly when administered in conjunction with GM-CSF (Fig. 10D). S100B addition also resulted in the increased survival and reduced size of human primary eosinophils. As shown in Fig. 10E–G, these attributes were significantly evident upon GM-CSF treatment but were further enhanced by the addition of S100B, which may, in part, be reflective of cell:cell interactions during culture above agarose versus non-specific adhesion and activation known to occur when cells are cultured on polystyrene (86). In addition, the S100B protein is known to interact with components of the cytoskeleton and modulate cell signals associated with cell morphology (87). The uniformity in size as shown in Fig. 10E–G suggests potential changes in the cytoskeleton and granule compartments that encourage the retention of granules (low side scatter), viability (lack of PI stain) and a morphologic decrease in size (low forward scatter). These characteristics are similar to identified BAL hyperdense eosinophils found after allergen challenge in a guinea pig model (88). In the antigen-challenged microenvironment, release of GM-CSF and S100B by T cells (8, 89) or certain tumor cells (90–93) may encourage the accumulation of eosinophils as has been identified in the asthmatic lung (94) and tumors of the colon (95) and skin (6). These observations suggest that in the presence of GM-CSF and S100B alone, cell signals in eosinophil degranulation are altered, thereby encouraging persistent and chronic inflammation.

The S100B cytokine is secreted by T cells, astrocytes, oligodendrocytes, found in various isolated human tumor tissue samples and known to bind and activate RAGE (8, 30, 42, 96, 97). Approximately 7 g of S100B is expressed in a healthy 1.4 kg human brain (98). Inflammation and tumor formation are indicated to increase tissue-level expression and the release of S100B (93, 98, 99). Eosinophils have been indicated to migrate into the brain (100), infiltrate tumors (83) and associate with neurons (9). While the functions of tissue-associated eosinophils are not clearly known, a reparative process is suspected, particularly with reference to developing subdural hematomas (100). The interactions of eosinophils with S100B and additional proteins in the microenvironment at these sites may lead to eosinophil activation, degranulation and the release of fibrolytic (plasminogen) and cytotoxic (eosinophil cationic protein, eosinophil-derived neutrotoxin) proteins involved in wound repair and the clearance of damaged cells. Alternatively, eosinophils may release S100A8 and S100A9 and possibly other additional S100 proteins known to enhance tumor progression (101) and inflammation (16).

In summary, we have identified the expression of RAGE and RAGE ligand mRNA and protein in human primary blood eosinophils. We have also shown that eosinophils respond to a reported RAGE ligand, S100B. These findings enhance our understanding of eosinophil function and the evolution of disease and immunity.

**Supplementary data**

Supplementary data are available at International Immunology Online.

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