Contrasting Roles for the Receptor for Advanced Glycation End-Products on Structural Cells in Allergic Airway Inflammation versus Airway Hyperresponsiveness

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Running head: RAGE on structural cells controls allergic airway responses

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Abbreviations

AHR:	Airway hyperresponsiveness
APC:	Antigen-presenting cell
BAL:	Bronchoalveolar lavage
BMDC:	Bone marrow derived dendritic cell
DC:	Dendritic cell
EC:	Epithelial cell
HE:	Hematoxylin and eosin
HDM:	House dust mite
HMGB-1:	High-mobility group box 1
ILC2:	Group 2 innate lymphoid cell
MCh:	Methacholine
MNC:	Mononuclear cell
OVA:	Ovalbumin
PAS:	Periodic acid-Schiff
PBLN:	Peribronchial lymph node
RAGE:	Receptor for advanced glycation end-products
RL:	Lung resistance
Th2:	T helper type 2
TSLP:	Thymic stromal lymphopoietin

Abstract

The receptor for advanced glycation end-products (RAGE) is a multi-ligand receptor which belongs to the immunoglobulin superfamily. RAGE is reported to be involved in various inflammatory disorders, however, studies that address the role of RAGE in allergic airway disease are inconclusive. RAGE sufficient (RAGE+/+) and RAGE deficient (RAGE-/-) mice were sensitized to ovalbumin (OVA), and airway responses were monitored after OVA challenge. RAGE-/- mice showed reduced eosinophilic inflammation and goblet cell metaplasia, lower T helper type 2 (Th2) cytokine production from spleen and peribronchial lymph node mononuclear cells, and lower numbers of group 2 innate lymphoid cells (ILC2s) in the lung compared to RAGE+/+ mice following sensitization and challenge. Experiments using irradiated, chimeric mice showed that the mice expressing RAGE on radio-resistant structural cells but not hematopoietic cells developed allergic airway inflammation, however, the mice expressing RAGE on hematopoietic cells but not structural cells showed reduced airway inflammation. In contrast, absence of RAGE expression on structural cells enhanced innate airway hyperresponsiveness (AHR). In the absence of RAGE increased IL-33 levels in the lung were detected, and blockade of IL-33 receptor ST2 suppressed innate AHR in RAGE-/- mice. These data identify the importance of RAGE expressed on lung structural cells in the development of allergic airway inflammation, Th2 cell activation, and ILC2 accumulation in the airways. RAGE on lung structural cells also regulated

innate AHR, likely through the IL-33-ST2 pathway. Thus, manipulating RAGE represents a novel therapeutic target in controlling allergic airway responses.

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Key words: RAGE, allergic airway inflammation, airway hyperresponsiveness, asthma

Introduction

Allergic asthma is characterized by inflammatory airway obstruction and airway hyperresponsiveness (AHR) (4, 7). Airway inflammation in allergic asthma is associated with mucous membrane swelling and infiltration of cells, including eosinophils, T lymphocytes, and mast cells (4, 31). The progressive inflammation leads to airway fibrosis, hypertrophy of smooth muscle cells, bronchial wall thickening, increased mucous-producing goblet cells and obstruction of the airways (3, 12, 23). Allergen-specific memory T helper type 2 (Th2) cells are thought to play a central role in the development of these responses (32). Recently, group 2 innate lymphoid cells (ILC2s), newly identified innate immune cells with the ability to release Th2 cytokines (formerly termed natural helper cells), have been reported to induce and enhance Th2 allergic inflammation (8, 13, 32).

The receptor for advanced glycation end-products (RAGE) is a multi-ligand receptor that belongs to the immunoglobulin superfamily, and recognizes a variety of ligands, including high-mobility group box 1 (HMGB-1), S100 family of proteins, advanced glycation end-products (AGE), β-sheet fibrillar materials, and prions (10, 33, 37). The receptor is expressed as a single-chain transmembrane receptor on epithelial, neuronal, vascular and inflammatory cells, usually at low levels under homeostatic conditions. In contrast, especially in the lung, high basal levels of RAGE expression have been identified relative to expression in other tissues (10, 26, 33, 34, 37).

AGE and RAGE were initially reported to be involved in microvascular and macrovascular complications of diabetes mellitus, renal failure and peritoneal injury in long-term peritoneal dialysis patients (17, 30, 36). Recently, RAGE was implicated in the pathogenesis and progression of various chronic immune/inflammatory disorders (10, 33, 34). The RAGE/HMGB-1 pathway has also been reported to play an important role in acute lung injury (45) and fibrosing lung disease (15). Recently, serum levels of soluble RAGE, soluble forms of membrane RAGE, were shown to be decreased in patients with asthma (38). Using mouse models of allergen-induced airway disease, Milutinovic, et al demonstrated that allergen-induced airway inflammation in RAGE-/- mice were attenuated compared to wild-type mice (27), however, the mechanisms defining how RAGE mediated these responses were not defined.

In the present study, we investigated the role of RAGE in a model of allergen-induced disease using RAGE-deficient (RAGE-/-) mice. RAGE-/- mice showed decreased airway inflammation, reduced Th2 cytokine production and accumulation of ILC2 compared to RAGE-sufficient (RAGE+/+) mice. Experiments using chimeric mice revealed a requirement for RAGE expression on structural cells for development of allergic airway inflammation. In contrast, absence of RAGE on structural cells enhanced innate AHR. Thus, manipulating RAGE on structural cells may be beneficial for controlling asthmatic responses.

Materials and Methods

Animals

RAGE-/- mice were generated as described (30). Briefly, RAGE mutant mice were originally created using E14.1 ES cells (129 background). After the bone marrow chimeric mice were generated, they were crossbred with Cre-transgenic mice (CD-1 background) that transiently express Cre recombinase in eggs. The resultant RAGE-/- mice were then backcrossed to C57BL/6J (Charles River, Yokohama, Japan) for nine generations. Ten-week-old female RAGE-/- mice and their littermates (RAGE+/+ mice) were used in all experiments. RAGE-/- mice were viable and displayed normal reproductive fitness without a striking phenotype. When housed under SPF conditions, no spontaneous disease development was observed in the RAGE-/- mice for up to 6 months of age.

All experiments were performed in accordance with the National Institutes of Health guidelines. All procedures were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Okayama University (Okayama, Japan).

Experimental protocol (sensitization and airway challenge)

RAGE+/+ and RAGE-/- mice were sensitized with 20 μg of ovalbumin (OVA) (Grade V; Sigma Chemical Co., St. Louis, MO) emulsified in 2.25 mg of alum

(ImjectAlum; Pierce, Rockford, IL) by intraperitoneal injection on days 0 and 14. Mice were subsequently challenged by inhalation exposure to aerosols with OVA (1% in saline) via the airways using ultrasonic nebulizer for 20 min on days 28, 29 and 30. Forty-eight hours after the last challenge, AHR was measured as described below, followed by collection of samples for further analyses (16).

Determination of airway responsiveness

Airway responsiveness was assessed by measuring changes in lung resistance (RL) in response to increasing doses of inhaled methacholine (MCh) (41) using a flexiVent small-animal ventilator (SCIREQ, Montreal, PQ, Canada). Before testing, mice were anesthetized by intraperitonial injection of pentobarbital (80 mg/kg), tracheostomized and mechanically ventilated. There were no significant differences in baseline values among the different groups.

Bronchoalveolar Lavage (BAL)

Immediately after assessment of airway function, lungs were lavaged via the tracheal tube with Hanks' balanced salt solution (HBSS) (2 x 1 ml, 37°C). The volume of the collected BAL fluid was measured in each sample, and the number of cells in BAL fluid was counted. Cytospin slides were stained with May-Giemsa and differentiated in a blinded fashion by counting at least 200 cells under light microscopy.

158

159 **Lung histology**

160 Lungs were fixed in 10% formalin and cut around the main bronchus and
161 embedded in paraffin blocks. The slides were stained with hematoxylin-eosin (HE) and
162 periodic acid Schiff (PAS) for identification of mucus-containing cells, and were
163 examined under light microscopy. In HE-stained lung sections, the numbers of
164 inflammatory cells per square millimeter in the peribronchial and perivascular tissue
165 were analyzed using the NIH Image Analysis system for quantitative evaluation. More
166 than 10 bronchioles in a minimum of 10 high-power fields per lung were randomly
167 examined in a blinded fashion. The numbers of mucus-containing cells (goblet cells)
168 were counted in more than 8 bronchioles in 10 high-power fields per animal by
169 measuring the length of epithelium defined along the basement membrane and luminal
170 area using the NIH Image Analysis system (11, 20).

171

172 **Culture of mononuclear cells (MNCs) from spleen and peribronchial lymph nodes**
173 **(PBLNs)**

174 Spleens of OVA-sensitized and challenged mice were removed and placed in
175 PBS (11). Cells were dispersed and MNCs were separated by a density gradient cell
176 centrifugation using Histopaque (Sigma- Aldrich, St. Louis, MO). Cells were washed,
177 counted and resuspended to a fixed concentration in RPMI 1640 (Wako Pure Chemical

178 Industries, Osaka, Japan) containing heat-inactivated 10% FCS and penicillin/
179 streptomycin. PBLNs were similarly manipulated. Cells (4×10^5) were plated in each
180 well of 96-well round-bottom plates, cultured at 37°C in a 5% CO_2 atmosphere in the
181 presence or absence of 10μg/ml OVA. Forty-eight hours after the last challenge, the
182 supernatants were removed and cytokine levels analyzed by ELISA as described below.
183
184 **Measurement of cytokines and chemokines**
185 Cytokine levels in the BAL fluid were measured by ELISA. All cytokines and
186 chemokines ELISAs were performed according to the manufacturer's directions. The
187 limits of detection were 1 ng/ml for HMGB-1, 7 pg/ml for IL-5, 1.5 pg/ml for IL-13, 3
188 pg/ml for IL-1β, 2 pg/ml for KC, 1.5 pg/ml for MIP-2, 2.8 pg/ml for IL-33, 0.71 pg/ml
189 for TSLP and 15 pg/ml for IL-25. All kits except for IL-25 (BioLegend, San Diego,
190 CA) and HMGB-1 (Shino-Test Corporation, Sagamihara, Japan) were from R&D
191 Systems (Minneapolis, MN). Lung homogenates were prepared as described (22).
192
193 **Lung cell isolation**
194 Lungs of OVA-sensitized and challenged mice was separated from the
195 associated lymph nodes, removed and placed in PBS containing heat-inactivated 10%
196 FCS. Lung tissue was minced and incubated for 1 h at 37°C in 5 ml PBS containing
197 0.05% collagenase I (Sigma-Aldrich), then lung tissue was dispersed by passing through

198 a 20 G needle several times and the suspensions were strained through a cell-strainer.
199 The pulmonary MNCs were isolated by density gradient cell centrifugation over
200 Histopaque (Sigma-Aldrich) (22).
201
202 **Flow cytometry**
203 Cells were incubated with PerCP or APC-conjugated anti-CD3, PE-conjugated
204 anti-CD8, FITC-conjugated anti-CD4 antibodies (BD Biosciences, San Diego, CA), and
205 then analyzed by flow cytometry on MACSQuant Analyzers (Miltenyi Biotec, Bergisch
206 Gladbach, Germany).
207
208 **Intracellular cytokine staining**
209 Lung MNCs were stimulated for 5 h with PMA (10 ng/ml) and ionomycin (500
210 μg/ml) in the presence of brefeldin A (10μg/ml). After washing, cells were stained for
211 cell surface markers with mAbs against CD3 (145-2C11, hamster IgG), CD4 (RM4-5,
212 rat IgG2a), and CD8 (53-6.7, rat IgG2a). All fluorochrome-labeled mAbs were
213 purchased from BD Biosciences. After fixation and permeabilization, cells were stained
214 with PE- or FITC-conjugated anti-cytokine antibodies purchased from BD Biosciences.
215 Staining was monitored by flow cytometry on MACSQuant Analyzers. The number of
216 cytokine-producing CD4, CD8 T cells per lung was calculated from the percent of
217 cytokine-producing cells and the number of CD4 or CD8 T cells isolated from the lung.

Analyses of ILC2s

The cells isolated from digested lungs were stained with biotin-conjugated antibody mixtures for lineage markers (CD4, CD5, CD8, CD11c, CD11b, CD19, NK1.1, Gr-1, TER119, FcεRI and B220), Pacific blue-conjugated anti-Sca-1, PECy7-conjugated c-Kit (CD117), APC-conjugated anti-IL-7Rα (CD127), FITC-conjugated anti-T1/ST2, APCCy7-conjugated anti-CD25 and PE-conjugated anti-streptavidin, and analyzed using MACSQuant Analyzer. $Lin^{-}Sca^{+}c\text{-}Kit^{+}IL\text{-}7R\alpha^{+}CD25^{+}ST2^{dim}$ cells were identified as lung ILC2s (18). The data were analyzed by FlowJo (TreeStar, Ashland, OR). APC-Cy7-conjugated anti-CD25, pacific blue-conjugated anti-Sca-1, biotin- conjugated anti-CD4, anti-CD5, anti-CD8, anti-CD11b, anti-NK1.1, anti-Gr-1, anti-TER119, anti-B220 and PE-conjugated anti-streptavidin were obtained from BD Biosciences. FITC-conjugated anti-T1/ST2 was from MD Bioscience (St Paul, MN). APC-conjugated anti-IL-7Rα and biotin-conjugated anti-FcεRI were from BioLegend (San Diego, CA). PECy7-conjugated c-Kit was from eBioscience (La Jolla, CA). Biotin-conjugated anti-CD11c and anti-CD19 were from TONBO biosciences (San Diego, CA).

Immunohistochemistry

Paraffin sections (5μm thick) were cut and mounted on poly-L-lysine-coated

glass slides. After removing the paraffin, endogenous peroxidases were quenched with 3% H_2O_2 in methanol. After washing, sections were incubated with rat monoclonal anti-RAGE or isotype-matched control antibody (R&D Systems), and incubated for another 20 minutes with Histofine Simple Stain MAX-PO (Nichirei Biosciences, Tokyo, Japan). The sections were immersed in diaminobenzidine as a chromogen for two minutes, counterstained with hematoxylin, and mounted under coverslips.

Generation of bone marrow chimeras

Femurs and tibias were obtained from 8 to 12-wk-old RAGE+/+ and RAGE-/- donor mice and bone marrow cells were harvested as previously described (42). Seven-to 10-wk-old RAGE+/+ and RAGE-/- recipient mice respectively received 12 Gy total body irradiation (TBI) on day -56. After TBI, $8x10^6$ BM derived cells from donor mice were injected intravenously into recipients on the same day.

For assessment of bone marrow cell homing and differentiation, transplantation with the same method using $CD45.1^+$ donor mice and $CD45.2^+$ recipient mice was performed. The spleen MNCs, cells of PBLNs and alveolar macrophages from BAL fluid were obtained. The degree of chimerism of $B220^+$ B cells, $CD11c^+$ dendritic cells, lung ILC2s, and $CD11b^+$ alveolar macrophages, known to be slowly repopulated following irradiation, was confirmed by staining of CD45.1 or CD45.2. Spleen MNCs, PBLN MNC and alveolar macrophages from BAL fluid were obtained and stained with

APC-labeled anti-CD11b, anti-CD11c, anti-B220, FITC-labeled anti-CD45.1, and PE-labeled anti-CD45.2 antibodies (BD Biosciences), and analyzed by flow cytometry.

Total RNA isolation and quantitative real-time PCR

The left lung was homogenized and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), treated with DNase (Qiagen, Valencia, CA) according to the manufacturers' instructions. Reverse transcription was performed using oligo (dT) primers and the Invitrogen Superscript II Reverse Transcriptase (Life Technologies, Grand Island, NY) to obtain cDNA for PCR. Quantitative real-time PCR was performed in a 25 μl reactions using SYBR Green master mix (Applied Biosystems, FosterCity, LA) and the StepOnePlus Real-Time PCR system (Applied Biosystems). Primers sequences were as follows: RAGE, forward, 5-ACTACCGAGTCCGAGTCTACC-3, and reverse, 5-GTAGCTTCCCTCAGACACACA-3; GM-CSF, forward, 5-GCGCCTTGAACATGACAGC-3, and reverse, 5-GGCTGTCTATGAAATCCGC-3; GAPDH, forward, 5-TATGTCGTGGAGTCTACTGGT-3, and reverse, 5-GAGTTGTCATATTTCTCGTGG-3. Relative expression levels for each target were normalized to GAPDH and calculated using the ΔΔ cycle-threshold method. There were no changes in GAPDH expression among groups.

Generation of bone marrow–derived dendritic cells (BMDCs)

Bone marrow cells obtained from femurs and tibias of RAGE+/+ or RAGE -/- mice were cultured and pulsed with recombinant mouse GM-CSF (10 ng/ml; R&D Systems) and recombinant mouse IL-4 (10 ng/ml; R&D Systems) on days1 and 5. On day 8, cells were pulsed with OVA (200 μg/ml, grade V; Sigma-Aldrich) for 24 h and washed three times with PBS. More than 90% of the cells were determined to be myeloid dendritic cells (DCs) ($CD11c^+$, $CD11b^+$, $Gr\text{-}1^-$) (28).

Transfer of BMDCs

Under anesthesia, $1x10^6$ OVA-pulsed BMDCs in 40μl of PBS were instilled into naïve RAGE+/+ through the trachea under fiberoptic illumination (28). Control groups of mice received OVA-non-pulsed BMDCs. Ten days after BMDC transfer, mice were exposed to aerosolized OVA (1% in saline) for 20 min/day for three consecutive days; 48 hours after the last challenge, AHR was assessed and BAL fluid was obtained.

Blockage of IL-33 receptor ST2

Rat anti-mouse ST2/IL‑1 R4 monoclonal (anti-ST2) antibody (40μg; R&D Systems) or isotype-matched control antibody (R&D Systems) in 40μl of PBS was administered intratracheally to RAGE+/+ or RAGE-/- mice under anesthesia, and 12 hours later, AHR was measured.

298

299 **Statistical analysis**

300 All results were expressed as the means ± SEM. ANOVA was used to determine
301 the levels of difference between all groups. Pairs of groups of samples distributed
302 parametrically were compared by unpaired 2-tailed Student *t* test, and those samples
303 distributed nonparametrically were compared by Mann-Whitney U test. Significance
304 was assumed at p values of <0.05.

305

306

Results

Allergic inflammation in the airways is decreased in RAGE-/- mice

Numbers of inflammatory cells in BAL fluid were assessed 48 hours after the last OVA challenge in RAGE+/+ and RAGE-/- mice. In sensitized and challenged (OVA/OVA) mice, total cells, lymphocytes and eosinophils were significantly increased compared with non-sensitized but challenged (PBS/OVA) mice. However, numbers of eosinophils were significantly lower in the BAL fluid of RAGE-/- mice compared to RAGE+/+ mice (Fig. 1A).

Inflammatory cell infiltration of the lungs was further investigated by histological examination. In HE-stained lung sections from RAGE-/- mice, lower numbers of inflammatory cells were detected following sensitization and challenge (Fig. 1B, C). The numbers of PAS-positive goblet cells were also significantly lower in RAGE-/- mice compared to RAGE+/+ mice following sensitization and challenge with OVA (Fig. 1D, E).

Cytokine levels in the airways

We measured cytokine levels in the BAL fluid by ELISA. OVA sensitization and challenge resulted in significant increases in IL-4, IL-5 and IL-13 levels in RAGE+/+ mice. In contrast, RAGE-/- mice showed significantly lower levels of IL-4, IL-5 and IL-13 following sensitization and challenge (Fig. 2A). The levels of KC,

MIP-2, and IL-1β were also lower in RAGE-/- mice compared to RAGE+/+ mice (Fig. 2B).

Spleen and PBLN MNCs from RAGE-/- mice release lower levels of Th2 cytokines

To determine if the attenuated Th2 cytokine secretion observed *in vivo* in RAGE-/- mice was due to impaired Th2 cytokine production, we assessed cytokine production in spleen and PBLN MNCs *in vitro*. The levels of IL-5 and IL-13 from OVA-restimulated spleen MNCs of RAGE-/- mice were significantly lower compared to RAGE+/+ mice (Fig. 2C). There were no significant differences in IL-17 and IFN-γ levels in the two strains of mice (data not shown). IL-5 and IL-13 levels from the PBLN MNCs were lower in RAGE-/- mice compared to RAGE+/+ mice (Fig. 2D). These data suggest that RAGE contributes to not only systemic sensitization of Th2 cells but also activation of Th2 cells in the airways.

Numbers of Th2 cells in the lungs

To determine if the accumulation of T cells in the airways of sensitized and challenged mice was affected by expression of RAGE, we assessed the numbers of T cells and cytokine-producing T cells in the lungs. There were no differences between the two strains of mice in numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T cells (data not shown). The numbers of $CD4^+$IL-13^+ cells and $CD4^+$IL-5^+ cells in the lungs were not lower in

RAGE-/- mice despite the lower levels of IL-5 and IL-13 in BAL fluid from sensitized and challenged RAGE-/- mice (Fig. 3A). There were also no significant differences in IL-17$^+$ and IFN-γ^+ cells in the two strains of mice (data not shown).

The numbers of ILC2s in the lungs of RAGE-/- mice were lower compared to RAGE+/+ mice following sensitization and challenge

Recently, ILC2s have been reported to contribute to allergic airway inflammation independent of Th2-cell mediated immunity (8, 14, 24). We assessed numbers of ILC2s in the airways of RAGE+/+ and RAGE-/- mice following sensitization and challenge with OVA. Interestingly, the numbers of ILC2s in the lungs of RAGE-/- mice were significantly lower compared to RAGE+/+ mice following sensitization and challenge (Fig. 3B). These data suggest that RAGE contributes to the accumulation of ILC2s in the airways in this model of OVA-induced allergic airway inflammation.

RAGE expression in lung tissues

The distribution of RAGE in lung tissue was determined by immunohistochemisty. Many RAGE+ cells were found in the alveolar wall areas of non-sensitized RAGE+/+ mice (Fig. 4A). OVA-sensitized and challenged RAGE+/+ mice also showed many RAGE+ cells in alveolar wall areas, similar to non-sensitized

mice. The *RAGE* mRNA levels in sensitized and challenged RAGE+/+ mice were not different from non-sensitized RAGE+/+ mice (Fig. 4B), suggesting that RAGE is spontaneously expressed in the lung and expression levels were not affected by sensitization and challenge.

RAGE expression on radio-resistant structural cells contributes to allergic airway inflammation

To determine the relative contribution of RAGE signaling on lung structural cells versus hematopoietic cells following allergen sensitization and challenge, we generated radiation-induced bone marrow chimeric mice. Eight weeks after reconstitution, chimerism was confirmed by flow cytometry in lymph node B lymphocytes, DCs, lung ILC2s, and alveolar macrophages.

The populations of inflammatory cells in the BAL fluid from the bone marrow chimeric mice following sensitization and challenge were evaluated (Fig. 5A). RAGE+/+ → RAGE+/+ mice had significantly increased numbers of eosinophils in the BAL fluid compared to RAGE-/- → RAGE-/- mice, but this response was markedly reduced in RAGE+/+ → RAGE-/- animals. In contrast, RAGE-/- → RAGE+/+ mice had increased numbers of eosinophils in BAL fluid, comparable to RAGE+/+ → RAGE+/+ mice.

In HE-stained lung sections of RAGE-/- → RAGE+/+ mice and RAGE+/+ → RAGE+/+ mice, accumulation of inflammatory cells following sensitization and challenge was detected. In contrast, very few cells were detected in RAGE-/- → RAGE-/- mice and RAGE+/+ → RAGE-/- mice (Fig. 5B, C). The numbers of PAS-positive cells in RAGE-/- → RAGE+/+ mice and RAGE+/+ → RAGE+/+ mice were also significantly higher compared to RAGE-/- → RAGE-/- mice and RAGE+/+ → RAGE-/- mice (Fig. 5D, E). IL-13 levels in BAL fluid of RAGE+/+ → RAGE-/- mice were significantly lower compared to RAGE+/+ → RAGE+/+ mice (Fig. 5F). In contrast, RAGE-/- → RAGE+/+ mice showed increased IL-13 levels comparable to RAGE+/+ → RAGE+/+ mice. These data suggested that expression of RAGE on structural cells is important for the full development of allergic airway inflammation and Th2 cytokine production in the airways following sensitization and challenge.

In vitro-allergen-pulsed RAGE-/- DCs induce eosinophilic inflammation

Our results using bone marrow chimeric mice suggested that RAGE expression on hematopoietic cells was less important than RAGE expression on radio-resistant structural cells. The influence of RAGE expression on DC function has also been reported (9). To directly evaluate the role of RAGE expression on DCs, we investigated the ability of antigen-pulsed BMDCs to induce allergic airway responses after intratracheal instillation. BMDCs from RAGE+/+ or RAGE -/- mice were pulsed with

406 OVA and instilled intratracheally into RAGE +/+ mice, followed by aerosolized OVA
407 challenge (11). These experiments showed that OVA-pulsed RAGE+/+DCs and
408 RAGE-/- DCs were comparable in inducing increases in eosinophilic inflammation,
409 confirming the functional competence of *in vitro* OVA-pulsed RAGE-/- DCs in
410 facilitating the development of allergic airway responses (Fig. 5G).
411
412 **IL-33, thymic stromal lymphopoietin (TSLP) and IL-25 levels in the lungs**
413 Cytokines and chemokines produced by airway epithelial cells (ECs) such as
414 IL-33, TSLP and IL-25 play important roles in both adaptive Th2 cell-mediated
415 immunity but also in innate immunity including activation of ILC2s for induction of
416 allergic airway responses (6, 18, 21, 43), therefore, we assessed levels of these
417 cytokines in lung tissue. However, the levels of IL-33, TSLP, and IL-25 were not
418 different in the two strains of mice after sensitization and challenge (Fig. 5H). IL-33
419 levels in challenged only RAGE-/- mice were significantly higher compared to
420 challenged only RAGE+/+ mice (Fig. 5H).
421
422 **AHR in RAGE+/+ and RAGE-/- mice after sensitization and challenge**
423 We then monitored AHR following sensitization and challenge with OVA.
424 Intraperitoneal OVA sensitization and airway challenge led to the development of
425 increased AHR in RAGE+/+ mice, as shown by significant increases in RL compared to

non-sensitized but challenged mice (Fig. 6). Challenged-only RAGE-/- mice showed significantly higher AHR compared to challenged-only RAGE+/+ mice. AHR in sensitized and challenged RAGE-/- mice was not higher than challenged-only RAGE-/- mice. Thus, AHR in RAGE-/- mice was not further increased after sensitization and challenge.

Naïve RAGE-/- mice demonstrate increased AHR

We next assessed AHR to increasing doses of inhaled MCh in naïve RAGE-/- mice and naïve RAGE+/+ mice. RAGE-/- mice showed significantly higher (baseline) MCh-responsiveness compared to RAGE+/+ mice (Fig. 7A).

Absence of RAGE expression on radio-resistant structural cells up-regulates AHR

To determine whether the expression of RAGE on radio-resistant structural cells or on hematopoietic cells contributed to innate AHR, we utilized the irradiated bone marrow chimeric mice. RAGE-/- → RAGE-/- mice showed significantly higher AHR compared to RAGE+/+ → RAGE+/+ mice. RAGE+/+ → RAGE-/- mice also showed significantly higher AHR compared to RAGE+/+ → RAGE+/+ mice, whereas RAGE-/- → RAGE+/+ mice showed significantly lower AHR compared to RAGE-/- → RAGE-/- and RAGE+/+ → RAGE-/- mice (Fig. 7B). These data suggested that

absence of RAGE expression on radio-resistant structural cells results in increased AHR.

Blockage of IL-33 receptor ST2 attenuates innate AHR in RAGE-/- mice

Experiments using chimeric mice showed that absence of RAGE expression on radio-resistant structural cells enhanced innate AHR. Because challenged only RAGE-/- mice showed higher IL-33 levels in the lung compared to RAGE+/+ mice (Fig. 5G), we assessed IL-33 levels in naïve mice, and confirmed that IL-33 levels in the lungs of naïve RAGE-/- mice were higher than in naïve RAGE+/+ mice (Fig. 7C). These data suggested that high levels of IL-33 in the lung might have contributed to innate AHR in RAGE-/- mice. To verify this hypothesis, we investigated whether blockade of IL-33 receptor ST2 attenuated AHR in RAGE-/- mice. RAGE-/- mice treated with anti-ST2 antibody showed significantly lower AHR compared to mice which received control antibody, suggesting that IL-33 in the lung was a key regulator of AHR in naïve RAGE-/- mice (Fig. 7D).

Discussion

In the present study, we demonstrated that RAGE contributes to both systemic sensitization and local activation of Th2 cells, as well as the accumulation of ILC2s in the airways following sensitization and challenge with allergen. Spleen MNCs as well as PBLN MNCs from RAGE-/- mice showed impaired Th2 cytokine production. Accumulation of ILC2s in the airway was reduced in the absence of RAGE although the numbers of Th2 cells were not different in the two strains of mice following sensitization and challenge. We demonstrated for the first time, to the best of our knowledge, that RAGE expression on radio-resistant structural cells plays a critical role in the full development of allergen-induced airway inflammation. Although RAGE is expressed on both hematopoietic and structural cells, we show in chimeric mice that the mice expressing RAGE on structural cells but not hematopoietic cells develop allergic airway inflammation.

Recently, ILC2s, newly identified innate immune cells with the capacity for Th2 cytokine production in response to airway EC-derived IL-25, IL-33, and TSLP, have been reported to induce innate immune responses and enhance Th2 allergic inflammation (8, 13, 32). Previous studies have focused on the role of protease-allergens such as papain, house dust mite (HDM) and cockroach to induce ILC2s. In the present study, we demonstrated that RAGE might contribute to accumulation of ILC2s in the lungs of OVA sensitized and challenged mice. Halim et al

(14) demonstrated that OVA together with IL-33 induces migration of Th2 cells to regional lymph nodes in the presence of ILC2s. In the present study, numbers of ILC2s in the lungs of RAGE-/- mice were significantly lower compared to RAGE+/+ mice. Therefore, RAGE may contribute to accumulation of ILC2s in the airways, and Th2 cells may collaborate with ILC2s to trigger allergic airway responses through RAGE signaling.

It has been reported that RAGE is constitutively expressed at high levels on lung structural cells (33). In contrast, there was relatively low expression of RAGE on vascular endothelial cells and inflammatory cells including neutrophils, monocytes/macrophages, lymphocytes, and DCs (33). Using chimeric mice, we demonstrated that the expression of RAGE, especially on radioresistant structural cells, was essential to the development of allergen-induced airway inflammation. Airway structural cells including ECs have been reported to be essential controllers of inflammatory, immune and regenerative responses to allergens, viruses and environmental pollutants that contribute to asthma pathogenesis (23). ECs express many pattern recognition receptors including RAGE and toll-like receptors (TLR) (23, 33, 37). EC triggering of TLR4 by HDM induced production of TSLP, GM-CSF, IL-25 and IL-33 (23). These innate cytokines have been implicated in the development of allergic airway inflammation and played a critical role in enabling antigen-presenting cells (APCs) to sensitize Th2 cells (2, 5, 6, 21, 25, 39, 43, 44). In the present study, RAGE-/-

503 mice were less responsive to allergen challenge, and somewhat similar to the role of
504 TLR4, RAGE on structural cells may contribute to secretion of IL-33, IL-25 and TSLP,
505 and increase APC sensitization of Th2 cells. These innate cytokines have also been
506 implicated in induction of ILC2s (8, 13). In addition to ECs, alveolar cells were reported
507 to have the capacity to secrete innate cytokines such as IL-33 (19). Therefore, RAGE
508 expression in alveolar cells may also induce activation of Th2 cells and accumulation of
509 ILC2s in the airways, and as a result amplify the full development of allergic airway
510 inflammation.

511 DCs, representative of lung APCs, are critical to the activation of lung immune
512 responses (35). Dumitriu, et al. reported the importance of RAGE for DC maturation (9).
513 During the preparation of this manuscript, Ullah, et al (40) reported that numbers of
514 DCs in the airway following intranasal instillation of HDM were lower in RAGE-/-
515 mice compared to RAGE+/+ mice, suggesting the importance of RAGE expression on
516 DC for sensitization. However, they did not compare RAGE-/- DCs with RAGE+/+
517 DCs for the ability to trigger airway inflammation. In the present study, using OVA as
518 an allergen, we demonstrated that RAGE-/- DCs were able to induce allergic airway
519 inflammation comparable to RAGE+/+ DCs, which is consistent with the previous
520 study showing APC function of RAGE-/- DCs (29). Although the allergens used were
521 different, at least in our model, OVA-pulsed DCs did not require RAGE expression for

allergic inflammation, confirming the importance of RAGE expression on structural cells but not hematopoietic cells.

Moser et al have reported that RAGE was required for *in-vivo* CD4 T cell proliferation using RAGE-/- OT-II cells (29). They have also recently shown that RAGE deficiency resulted in reduction of IL-5 and IFN-γ, and enhancement of IL-17 production from OT-II cells following OVA challenge, which was associated with lower accumulation of OT-II cells in the airway (1). In the present study, we have demonstrated lower IL-5 and IL-13 production from spleen and PBLN cells of RAGE -/-mice suggesting a requirement for RAGE for systemic Th2 sensitization *in vivo*. We also saw lower Th2 cytokine levels in BAL fluid, but did not see differences in numbers of Th1, Th2, and Th17 cells in the airways between RAGE-/- and RAGE+/+ mice. Direct comparison of their studies and the present study is difficult because of differences in experimental protocols. Using chimeric mice, we have clearly shown the critical role of RAGE expression on radio-resistant structural cells but not T cells for the development of allergic airway inflammation and Th2 cytokine secretion in the airway. We have also shown the importance of RAGE expression on structural cells in elastase-induced airway inflammation and emphysematous change in mice (42).

RAGE-/- mice, even in a naïve state showed significantly higher MCh-induced AHR compared to RAGE+/+ mice. Using a different strain of RAGE-/- mice, Milutinovic and colleagues reported no significant differences in AHR, although

RAGE-/- mice trended to higher AHR compared to wild-type mice. We further demonstrated the expression of RAGE on radio-resistant lung structural cells as perhaps the major contributor to the increased AHR in naïve mice. Interestingly, levels of IL-33 in the lungs of RAGE-/- mice were significantly higher than in RAGE+/+ mice. We demonstrated that blockade of ST2 attenuated innate AHR in RAGE-/- mice. Thus absence of RAGE appears linked to increased IL-33 levels in the lung, perhaps from EC, which might, at least in part, have contributed to the development of increased AHR in non-sensitized and non-challenged RAGE-/- mice. The mechanism(s) whereby the absence of RAGE leads to increased IL-33 levels in the lungs is not currently defined.

In summary, we demonstrated that RAGE played a critical role in airway inflammation, activation of Th2 cells, and the accumulation of ILC2 in the airways. Further, we characterized the importance of RAGE expression on structural cells in the development of airway inflammation. In contrast, absence of RAGE on structural cells enhanced innate AHR. Our data suggest that manipulation of these contrasting roles of RAGE on structural cells for allergic airway inflammation versus AHR may be beneficial for controlling asthmatic responses.

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733 **Figure Legends**

734 **Figure 1.** RAGE-/- mice develop reduced airway inflammation following sensitization
735 and challenge. (A) Cellular composition in bronchoalveolar lavage (BAL) fluid. Data
736 represent the mean ± SEM (n=8-9 in each group). Significant differences (*: $P < 0.05$;
737 **: $P < 0.01$) between RAGE+/+ and RAGE-/- mice. #: $P < 0.01$ compared to
738 challenged only mice. (B) Hematoxylin and eosin-stained lung sections. Scale bar:
739 100μm. (C) Inflammatory cell numbers in the peribronchial and perivascular tissue (n=4
740 in each group). Significant differences (*: $P < 0.05$; **: $P < 0.01$) between RAGE+/+
741 and RAGE-/- mice. #: $P < 0.05$ compared to challenged only mice. ##: $P < 0.01$
742 compared to challenged only mice. (D) Development of goblet cell metaplasia in the
743 airways of RAGE+/+ and RAGE-/- mice. Scale bar: 100μm. (E) Goblet cell metaplasia
744 was quantified in periodic acid Schiff (PAS)-stained sections as described in Materials
745 and Methods. Significant differences (**: $P < 0.01$) between RAGE+/+ and RAGE-/-
746 mice. #: $P < 0.01$ compared to challenged only mice.

747

748 **Figure 2.** Cytokine levels and numbers of cytokine producing cells in the airways and T
749 helper type 2 (Th2) cytokine production from spleen and peribronchial lymph node
750 (PBLN) mononuclear cells (MNCs). (A) Th2 cytokine levels (IL-4, IL-5 and IL-13),
751 and (B) neutrophil related chemokines (KC and MIP-2), and IL-1β in BAL fluid were
752 measured by ELISA as described in Materials and Methods. The results for each group

753 are expressed as means ± SEM (n=6-8 in each group). Significant differences (*: P <
754 0.05; **: P < 0.01) between RAGE+/+ and RAGE-/- mice. #: P < 0.05 compared to
755 challenged only mice. ##: P < 0.01 compared to challenged only mice. (C) IL-5 and
756 IL-13 levels in supernatants from spleen cultured in the presence or absence of
757 ovalbumin (OVA) (10 mg/ml) determined by ELISA (n=7-8 in each group). *: P < 0.05.
758 (D) IL-5 and IL-13 levels in supernatants from PBLNs cultured in the presence or
759 absence of OVA (n=4 in each group). *: P < 0.05.
760
761 **Figure 3.** Numbers of T cells and group 2 innate lymphoid cells (ILC2s) in the lung
762 following sensitization and challenge. (A) Numbers of IL-13 and IL-5 producing $CD4^+$
763 T cells in the lung after sensitization and challenge. Numbers of cells in the lung were
764 determined as described in Materials and Methods. Data represent the mean ± SEM
765 (n=7 in each group). There were no significant differences between RAGE+/+ and
766 RAGE-/- mice. (B) Numbers of ILC2 in the lungs of RAGE+/+ and RAGE-/- mice
767 following sensitization and challenge (n=7 in each group). #: P < 0.01 compared to
768 challenged only mice. **: P < 0.01
769
770 **Figure 4.** RAGE expression in lung tissue. (A) RAGE expression was evaluated by
771 immunohistochemistry 48 hours after the last challenge with OVA as described in
772 Materials and Methods. RAGE+ cells are indicated by brown staining. RAGE

773 expression was found mainly on alveolar epithelial cells in both RAGE+/+ mice without
774 sensitization and RAGE+/+ mice following sensitization and challenge with OVA.
775 Scale bar: 100μm. (B) mRNA expression levels of RAGE in the lungs of RAGE+/+ and
776 RAGE-/- mice following sensitization and challenge (n=4 in each group).
777
778 **Figure 5.** RAGE expression on radioresistant structural cells contributes to allergic
779 airway inflammation. (A) Cellular composition in BAL fluid of chimeric mice (n=10-12
780 in each group). *: $P < 0.05$; **: $P < 0.01$. (B) Hematoxylin and eosin-stained lung
781 sections. Scale bar: 100μm. (C) Inflammatory cell numbers in the peribronchial and
782 perivascular tissue (n=4 in each group). *: $P < 0.05$; **: $P < 0.01$. (D) Development of
783 goblet cell metaplasia in the airways of chimeric mice. Scale bar: 100μm. (E) Goblet
784 cell metaplasia was quantified in periodic acid Schiff (PAS)-stained sections as
785 described in Materials and Methods (n=4-6 in each group). *: $P < 0.05$; **: $P < 0.01$. (F)
786 IL-13 levels in BAL fluid of chimeric mice measured by ELISA (n=12-14 in each
787 group). *: $P < 0.05$. (G) Cellular composition in BAL fluid of recipients of OVA-pulsed
788 dendritic cells (DCs). $1x10^6$ OVA-pulsed bone marrow-derived DCs from RAGE+/+ or
789 RAGE-/- mice (donor) were instilled intratracheally into naïve RAGE+/+ mice
790 (recipient), followed by challenge with OVA as described in Materials and Methods
791 (n=6 in each group). There were statistically no differences among the two groups. (H)
792 IL-33, thymic stromal lymphopoietin (TSLP) and IL-25 levels in the lung tissues

following sensitization and challenge with OVA compared to challenged only mice. RAGE+/+ and RAGE-/- mice were sensitized and challenged with OVA as described in Materials and Methods. The levels of these cytokines were measured by ELISA (PBS/OVA group: n=3-4, OVA/OVA group: n=6-9). Significant differences (*: P<0.01;) between RAGE+/+ and RAGE-/- mice. #: P < 0.05 compared to challenged only mice. ##: P < 0.01 compared to challenged only mice.

Figure 6. Airway hyperresponsiveness (AHR) in RAGE+/+ and RAGE-/- mice after sensitization and challenge with OVA. Forty-eight hours after the last challenge, lung resistance (RL) was monitored in response to increasing concentrations of inhaled methacholine (MCh), as described in Materials and Methods (n=9-11 in each group). *: P<0.05

Figure 7. Absence of RAGE expression on radio-resistant structural cells is associated with increased AHR. (A) AHR in naïve RAGE-/- and RAGE+/+ mice (n=14-16 in each group). *Significant differences (P < 0.05) between RAGE+/+ and RAGE-/- mice. (B) AHR in irradiated chimeric mice. The analysis was performed 8 weeks after bone marrow transplantation as described in Materials and Methods (n=5-7 in each group). *: P < 0.05. (C) IL-33 levels in the lungs of naïve mice, measured by ELISA (n=5 in each group). *: P<0.05. (D) Effects of IL-33 receptor ST2 blockade on innate AHR in

813 RAGE-/- mice. Twelve hours after intratrachial administration of anti-ST2 antibody or
814 control antibody, RL was monitored as described in Materials and Methods (n=4-5 in
815 each group). *: $P < 0.05$.
816