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Receptor for Advanced Glycation End Products Expression on T Cells Contributes to Antigen-Specific Cellular Expansion In Vivo

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Receptor for advanced glycation end products (RAGE) is an activation receptor triggered by inflammatory S100/calgranulins and high mobility group box-1 ligands. We have investigated the importance of RAGE on Ag priming of T cells in murine models in vivo. RAGE is inducibly up-regulated during T cell activation. Transfer of RAGE-deficient OT II T cells into OVA-immunized hosts resulted in reduced proliferative responses that were further diminished in RAGE-deficient recipients. Examination of RAGE-deficient dendritic cells did not reveal functional impairment in Ag presentation, maturation, or migratory capacities. However, RAGE-deficient T cells showed markedly impaired proliferative responses in vitro to nominal and alloantigens, in parallel with decreased production of IFN-γ and IL-2. These data indicate that RAGE expressed on T cells is required for efficient priming of T cells and elucidate critical roles for RAGE engagement during cognate dendritic cell-T cell interactions. The Journal of Immunology, 2007, 179: 8051–8058.

The ligands of receptor for advanced glycation end products (RAGE)† include members of the S100/calgranulin family, the nuclear protein high-mobility group box 1 (HMGB-1), and advanced glycation end products (the products of nonenzymatic glycation and oxidation of proteins and lipids). Rodent models have established a broadly protective role for pharmacological inhibitors of RAGE and its ligands in the acute (sepsis) and chronic inflammatory response (experimental allergic encephalitis), but the underlying mechanism(s) of protection remain mostly obscure.

The signal transduction pathway of RAGE, a member of the Ig superfamily, is incompletely understood, but engagement induces activation of small GTPases, MAPKs (p38 and ERK1/2), and NF-κB. Both RAGE and RAGE ligands, including S100/calgranulins and HMGB-1, are inducibly expressed at sites of inflammation (1–3). In particular, HMGB-1 is secreted by activated monocytes (4) and is released by dying cells (5). Importantly, wild-type but not HMGB-1−/− necrotic cells induced monocyte production of TNF-α, suggesting a critical role for HMGB-1 in the activation of the innate immune response by cellular necrosis (6). A broad contribution of RAGE and its ligands to the inflammatory response has been suggested because pharmacological blockade of RAGE or HMGB-1 attenuates acute septic inflammatory responses (4, 7), vascular diseases including atherosclerosis and diabetic vasculopathy (8), and T cell-mediated inflammatory responses including NOD diabetes (9), arthritis (10), experimental allergic encephalitis (11), and T cell-mediated allogeneic transplant rejection (12).

Mechanistic studies of RAGE and its ligands in the inflammatory responses have emphasized contributing roles in recruitment, adhesion, and migration of cellular innate effectors. Early studies noted that RAGE engagement induced up-regulation of endothelial adhesion markers. Neutrophil migration into microbial-laden peritoneal sites was impaired in RAGE−/− animals (7). With respect to adaptive immunity and T cell function, RAGE contributes to effect T cell responses in vivo as soluble (sRAGE) inhibits both the delayed-type hypersensitivity (DTH) response and the development of diabetes in NOD/SCID recipients of diabeticogenic T cells (9). In the experimental allergic encephalitis induced in mice by myelin basic protein immunization, treatment with sRAGE prevented disease and impaired recruitment of effector T cells into CNS sites. The specificity of sRAGE in distinct biological settings may be complex, because the persistent normal DTH response in RAGE-deficient mice suggests instead a limited role for RAGE in adaptive immunity (7). These studies (7) were performed using RAGE-deficient mice on a mixed genetic background, which may have produced variability in DTH responses, obscuring RAGE-specific contributions. In vitro studies with cultured dendritic cells (DC) have demonstrated that HMGB-1 is released by activated DCs and can act in a paracrine/autocrine manner modulating T cell activation (13).

The specific contributory roles of RAGE expressed by either T cells or APCs in the adaptive immune response remain unclear. Because it is important to identify the cellular targets of RAGE antagonists and blocking Abs responsible for their significant anti-inflammatory properties, we have investigated the role(s) of RAGE...
on T cells vs DCs using RAGE-deficient T cells and DCs in vitro and in vivo.

Materials and Methods
Mice
Homozgyous RAGE-null mice (14, 15) were backcrossed >10 generations into C57BL/6 before study and were mated with TCR-transgenic mice recognizing OVA residues 323-339 in the context of I-A<sup>d</sup> C57BL/6-Tg(TcraTcrb)25Scn (OT-II; originally provided by Alan Frey, New York University School of Medicine, New York, NY) and B6.SJL-congenic mice (B6.SJL-Ptprca/BoAiTac, termed B6.H2<sup>b</sup>, CD45.2<sup>+</sup>) to generate CD45.1<sup>+</sup> OT-II RAGE<sup>+</sup> and RAGE<sup>−/−</sup> mice. C57BL/6 (termed B6.H2<sup>b</sup>, CD45.2<sup>+</sup>) and BALB/cJ mice (H2<sup>d</sup>, CD45.2<sup>+</sup>) were purchased from The Jackson Laboratory. All animals were maintained in a temperature-controlled room with alternating 12-h light/dark cycles. All experiments were approved by the Institutional Animal Care and Use Committee of Columbia University. These studies conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, MD).

Abs, Ags, and other reagents
Anti-mouse CD8α-PerCP (Ly-2; 53-6.7), anti-mouse CD4-PerCP-Cy5.5 (Ly-3), anti-mouse CD44-APC (53-10.4), anti-streptavadin-PE, anti-mouse IL-2-PE (JES6-5H4), anti-mouse IFN-γ-AlexaFluor 647 (XMGL2), purified anti-mouse CD40 NA/LE (HM40-3), anti-mouse CD11c-APC (integrin αM; M1/70), anti-mouse CD11b-PerCP-Cy5.5 (Mac-1 HL3), anti-mouse CD45R/B220-PerCP-Cy5.5 (RA3-6B2), anti-mouse mouse IgG1, κ isotype control (anti-TNP) NA/LE were purchased from BD Pharmingen. The H-2D<sup>b</sup>-restricted 323–339 peptide (OVA<sub>257–264</sub> or OVA<sub>323–339</sub>) and OVA in 96-well plates coated with OVA at 90:1 ratio. For RT-PCR, total CD4<sup>+</sup> and CD8<sup>+</sup> was processed directly to cDNA synthesis activity of the Lmnausus amebocyte lysate kit (BioWhittaker) and were assessed directly for DC maturation activity (OVA and anti-OVA concentrations at 10 and 50 μg/ml, respectively). No reagent induced any change in the immunophenotype of DCs after 48 h of incubation. Recombinant murine fms-like tyrosine kinase-3 ligand (Flt3L) was a gift from Amgen. Hypoxanthine-guanine phosphoribosyltransferase. Hypoxanthine-guanine phosphoribosyltransferase.

Generation of bone marrow DCs (BMDC)
GM-CSF expanded BMDCs were prepared as described in Ref. 17. For expansion of BMDC with Flt3L, 2 × 10<sup>6</sup> cells/ml were plated in the presence of 200 ng/ml Flt3L. Cells were harvested on day 8 and incubated with LPS. CpG, or poly(I:C) or were left untreated until flow cytometric analysis 24 h later.

T cell proliferation studies
For in vitro and in vivo studies CD4<sup>+</sup> (OT-II) T cells were positively selected from pooled lymph node and splenocyte single-cell suspensions with anti-CD4 magnetic beads (Miltenyi Biotec). For in vivo proliferation studies, 5 × 10<sup>5</sup> CFSE-labeled CD45.1<sup>+</sup> OT-II T cells (in 200 μl) were injected into the tail vein (i.v.) of C57BL/6 RAGE<sup>−/−</sup> and C57BL/6 RAGE<sup>+/+</sup> mice (age and sex matched). After 24 h, recipient mice were injected i.p. with 150 μl of an emulsion of OVA (100 μg) in CFA/OVA or i.v. 1 × 10<sup>6</sup> washed OT-II peptide (10 μM)-pulsed GM-CSF BMDC. Mice were sacrificed on day 3 after T cell injection for flow cytometric CFSE dilution analysis, gating on the double-positive CD4<sup>+</sup> RAGE<sup>−/−</sup> population. In vitro CD4 proliferation studies were performed either by CFSE dilution analysis or thymidine incorporation as described.

Ag presentation assays to OT-I and OT-II hybridomas
BMDC (2.5 × 10<sup>5</sup>) were incubated with increasing concentrations of OVA:IC (90:10), peptide (OVA<sub>257–264</sub> or OVA<sub>323–339</sub>) or OVA in 96-well plates (18). After removal of the Ags, DCs were cocultured with 3 × 10<sup>5</sup> OT-I (MHC class I-restricted) or OTZ-II (MHC class II-restricted) T cell hybridomas. Activation of these T cell hybridomas induces β-galactosidase production, measured spectrophotometrically by cleavage of chlorophenol red-β-d-galactopyranoside (Calbiochem).

![FIGURE 1.](http://www.jimmunol.org/) RAGE is expressed by memory T cells and is induced in naive T cells upon TCR stimulation. A and B, RT-PCR of total splenic CD4<sup>+</sup> OT-II T cells isolated with magnetic beads (left). RT-PCR (A) and real-time PCR analysis (B) of CD4<sup>+</sup> (memory) and CD4<sup>+</sup> (naive) OT-II cells using RNA isolated from FACS-sorted splenocyte CD4<sup>+</sup> populations from OT-II mice. C and D, Real-time PCR results of RNA isolated at the indicated time points after stimulation of OT-II CD4<sup>+</sup> T cells on anti-CD3/ anti-CD28-coated plates (C) or by DCs (D) pulsed with OVA-containing immune complexes (50 μg/ml anti-OVA:10 μg/ml OVA). In the latter case, RAGE-deficient DCs were used for stimulation to prevent detection of RAGE transcripts from DCs. Data are expressed relative to unstimulated cells. HPRT, Hypoxanthine-guanine phosphoribosyltransferase.
Intracellular and soluble cytokine measurements

OT-II cells were cultured in the presence of peptide-pulsed BMDC as described above, and intracellular staining of cytokines IL-2 and IFN-γ by T cells was analyzed by flow cytometry according to protocols suggested by BD Pharmingen. Briefly, cells were first stained for cell surface CD4 Ag and then fixed with PBS containing 4% (v/v) paraformaldehyde, washed with permeabilization buffer containing 1% BSA and 0.5% saponin, and incubated with fluorochrome-conjugated anti-cytokine Abs. Cells were then washed with permeabilization buffer and analyzed on a FACSCalibur. Cytokines in cell culture supernatants were analyzed on a multiplex Luminox system. Thi/Th2 and inflammation multiplex microspheres (Luminex Corp.) from BioSource International were used to detect the cytokines IL-2, IL-4, IL-5, IFN-γ, IL-1β, IL-6, TNF-α, and IL-12.

DC migration in vivo

To assess migration of cutaneous DCs, tetramethylrhodamine-5-(and-6)isothiocyanate (5(6)-TRITC; Molecular Probes) was dissolved in a 1:1 v/v aceton-dibutylphosphate mixture just before application. Mice were painted on three areas on the dorsal surface of each side of the mouse on the shaved back skin with 25 µl of 8 mg/ml TRITC. Mice were sacrificed 18 h after skin painting, and the draining lymph nodes (inguinal, brachial, and axillary) were dissociated and digested in RPMI 1640 with Liberase Blendzyme 3 (Roche) and DNase I (Sigma-Aldrich) for 1 h at 37°C with intermittent agitation and analyzed by flow cytometry.

To assess in vivo migration of BMDCs, 5 × 10^6 CFSE-labeled BMDCs were injected into the footpads; mice were sacrificed 18 h later; and draining lymph nodes (popliteal, brachial, and axillary) were collected, dissociated, and digested as above and assessed by flow cytometry.

DCT cell conjugates

DCT cell conjugates were detected by flow cytometry using BMDC labeled with 5 µM PKH26 (Sigma-Aldrich) and OT-II CD4^+ RAGE^+ or RAGE^-^- T cells labeled with 5 µM CFSE (Molecular Probes). Before labeling, DC were incubated with 10 µM OT-II peptide for 5 h or left untreated. DCT cell conjugates were formed at a ratio of 1:5, pelleted at 50 × g for 5 min at room temperature, and cocultured in a humidified incubator at 37°C.

Statistical analysis

For comparing several groups, repeated measure ANOVA techniques were used. Further multiple comparisons were generated by mixed modeling methodology in SAS PROC MIXED. Student’s t test was used to compare 2 groups. p values were not adjusted for multiplicity of testing. For all statistical analyses, data were analyzed using the SAS system software (SAS Institute).

Results

RAGE is required for effective T cell priming in vivo

RAGE has been implicated in the activation of both innate and adaptive immune responses. Here, we specifically investigated whether RAGE expression on T cells contributes to initial T cell priming using I-A^b^-restricted OVA specific OT-II CD4 TCR-transgenic cells. RAGE was expressed in freshly isolated primary OT-II CD4 cells, initially down-regulated within 6 h after anti-CD3/anti-CD28 stimulation in vitro, and then up-regulated in OT-II CD4 T cells over 72 h. In vivo, RAGE was expressed preferentially in the previously activated CD44^- memory pool of primary CD4 and CD8 cells (Fig. 1).

Because RAGE is expressed on multiple hematopoietic lineage cells including APCs and T cells, it is conceivable that each might influence the immune response in vivo. Thus, we investigated the distinct contributions of RAGE on T cells and host non-T cells in adoptive transfer systems. To this end 5 × 10^6 CFSE-labeled RAGE^+^ and RAGE^-^- OT-II CD4^+^ CD45.1^-^- cells were transferred into syngeneic C57BL/6 CD45.2^-^- RAGE^+^ or RAGE^-^- hosts, and the mice were subsequently immunized i.p. with CFA-OVA. Homing of naive T cells to secondary lymphoid organs, including spleen and cervical nodes, was comparable in all immunized mice and thus was independent of RAGE expression on either host cells or OT-II cells (data not shown). Three days after immunization, isolated splenocytes were examined by flow cytometry to assess proliferative responses, enabled by the use of CFSE dilution analysis and the allogenic marker CD45.1 to specifically identify transferred OT-II cells (Fig. 2). Absence of RAGE on T cells substantially reduced T cell proliferative responses upon Ag exposure. In wild-type recipients, 27% of RAGE^+^- OT-II cells and 51% of RAGE^-^- OT-II cells remained undivided (Fig. 2B, p < 0.0001). RAGE expression on host cells also contributed to T cell priming as transferred RAGE^-^- OT-II cells divided more briskly in RAGE^+^- mice than in RAGE^null^ recipients (27% vs 42% undivided, respectively; p = 0.01). These effects were additive in that loss of RAGE on both host cells and transferred OT-II cells resulted in the lowest proliferative response (58% undivided).

These data indicate that RAGE contributes to quantitative early T cell expansion during T cell priming in vivo.

RAGE expression on T cells is required for APC-triggered initial activation

To directly examine the importance of RAGE on APCs and responding T cells, in vitro proliferation assays were performed using OT-II cells and DCs either containing or lacking RAGE. RAGE^+^ and RAGE^-^- OT-II cells proliferated normally in response to activation with plate-bound anti-CD3 (Fig. 3A), indicating that RAGE-deficient T cells do not have cell-autonomous defects in TCR-mediated activation responses. However, RAGE-deficient OT-II cells demonstrated impaired proliferative responses after stimulation with immature or LPS-matured OVA323–339 peptide-pulsed DCs (Fig. 3, B and C). Because RAGE-deficient T cells are fully capable of normal activation responses triggered by anti-CD3, these findings suggest that the requirement for RAGE on T cells is more likely due to engagement with RAGE ligands produced by APCs, rather than the T cells themselves.

To assess the contributions of RAGE expressed by DCs, RAGE^+^ or RAGE^-^- BMDCs pulsed with OVA323–339 were used to stimulate OT-II cells (Fig. 3B). OT-II CD4 cells proliferated comparably when stimulated with either RAGE^+^ or RAGE^-^- DCs, arguing against a strong contribution of RAGE in DC-mediated Ag presentation in vitro.

To address whether RAGE on T cells also contributed to allo-reactive responses, allogeneic T cell responses were assessed in vitro. RAGE^+^ or RAGE^-^- C57BL/6 MHCb splenic CD4 cells
RAGE regulates T cell activation

To assess whether RAGE-deficient T cells exhibited altered effector differentiation, OT-II RAGE$^{+/+}$ and RAGE$^{-/-}$ cells were expanded for 7 days with RAGE$^{+/+}$ or RAGE$^{-/-}$ DCs and cognate peptide; the cells were collected and then restimulated for 24 h with fresh RAGE$^{+/+}$ or RAGE$^{-/-}$ DCs and peptide and intracellular staining performed for IFN-$\gamma$ and IL-2 (Fig. 4A). Loss of RAGE on T cells but not on DCs significantly altered the production of IL-2 and IFN-$\gamma$ by responding T cells, consistent with a role for RAGE in Th-type polarization. Significantly fewer RAGE$^{-/-}$ OT-II cells produced IL-2 and IFN-$\gamma$, and these data were confirmed with fluorescent bead assays of cultured supernatants (Fig. 4B). Furthermore, analysis of the cytokines released into the supernatant demonstrated that RAGE$^{-/-}$ OT-II cells produced more Th2-type cytokines, IL-4 and IL-5 consistent with preferential Th2-type polarization of RAGE-deficient T cells.

RAGE-deficient DCs exhibit normal Ag processing and presentation capacity in vitro and in vivo

Previous reports suggested that RAGE expressed by DCs was important for DC maturation responses and their functional capacity to activate T cells. The ability of RAGE-deficient DCs to activate
OTZ-II cells were re-examined in vitro and in vivo. OVA<sub>323–339</sub>-pulsed RAGE<sup>+/+</sup> and RAGE<sup>-/-</sup> BMDCs were cocultured with CFSE-loaded RAGE<sup>+/+</sup> OT-II CD4<sup>+</sup> T cells in vitro or transferred into RAGE<sup>+/+</sup> OT-II CD4<sup>+</sup> T cells in vivo (left) or in vivo (right) was assessed flow cytometrically 3 days later. Representative data of the mean percent undivided OT-II cells in vivo are shown (top). The functional Ag-processing capacity of RAGE-deficient DC was examined using RAGE<sup>+/+</sup> or RAGE<sup>-/-</sup> DCs pulsed with whole OVA or OVA-containing immune complexes which are efficiently internalized and processed via activating FcγRs. These cells were coincubated with OVA-specific OTZ-I (K<sub>t</sub>-restricted) and OTZ-II (I-<sub>A</sub><sup>rl</sup>-restricted) hybridomas to respectively assess the cross-presentation and exogenous Ag-processing pathways. Both MHC class I-restricted and MHC class II-restricted T cell hybridomas were equivalently activated by Ag-pulsed RAGE<sup>+/+</sup> or RAGE<sup>-/-</sup> BMDCs (Fig. 5B). Furthermore, addition of LPS to these cultured DCs did not demonstrate any RAGE-dependent differences in Ag processing/presentation (data not shown). Thus, Ag processing and presentation to T cells are unaffected by deletion of RAGE in BMDCs.

**RAGE does not contribute directly to TLR-mediated activation of DCs**

Recent in vitro studies using HMGB-1 and RAGE-blocking Abs support a model that TLR-mediated activation of conventional and plasmacytoid DCs (13, 19) induces extracellular release of HMGB-1 which acts in an autocrine/paracrine manner to activate DCs. To identify a potential role for RAGE in DC activation, RAGE expression was examined in GM-CSF cultured DCs by real-time PCR after activation with LPS, anti-CD40, or with Ag:Ab immune complexes, three different classes of stimuli known to induce DC activation through TLR, TNF-receptor and immunoreceptor tyrosine activation signaling, respectively. Neither LPS nor anti-CD40 treatment up-regulated RAGE expression 24 h after treatment. However, incubation of BMDCs with immune complexes led to up-regulation of RAGE RNA transcripts in a manner that was inhibitable by HMGB-1 blocking Abs. These data are consistent with positive feedback regulation of RAGE RNA expression by its ligand HMGB-1 (Fig. 6A).
mals were examined at baseline and after injection with 10 ng of RAGE ligand HMGB-1 is released from DCs and can contribute to DC
To determine whether RAGE-deficient DCs are activated normally by TLR ligands, F1t-3 ligand-supported BMDCs were used to permit evaluation of both plasmacytoid (pDC) and conventional (cDC) subsets of DCs. F1t-3 ligand-supported cultures from RAGE+/+ and RAGE−/− bone marrow contained comparable numbers of both CD45R+ pDCs and CD45R− cDCs, indicating that RAGE did not influence DC development in vitro. Addition of TLR ligands (including LPS, poly(I:C), and CpG, which signal through TLR4, -3, and -9, respectively) to cDC cultures activated both RAGE+/+ and RAGE−/− cDCs equivalently as exhibited by similar TLR-induced immunophenotypic up-regulation of surface expression of CD86 and MHC class II (Fig. 6C) and by TLR-induced production of immunostimulatory cytokines (Fig. 6D). Furthermore, RAGE+/+ and RAGE−/− pDCs showed indistinguishable up-regulation of CD86 and MHC class II after TLR9 activation by CpG. To determine the role of RAGE in TLR-mediated activation of DCs in vivo, RAGE+/+ and RAGE−/− animals were examined at baseline and after injection with 10 μg of LPS. RAGE+/+ and RAGE−/− CD11c+ splenocytes contained comparable numbers of CD11c+ CD11b+ myeloid, and CD11c+ CD8− lymphoid DC populations (Fig. 6E, top). Twenty-four hours after injection with LPS, flow cytometric evaluation of CD86 expression demonstrated that CD86 was induced equivalently on RAGE+/+ and RAGE−/− CD11c+ splenic DCs (Fig. 6E, bottom). Thus, RAGE does not contribute directly to TLR-mediated activation of DCs in vitro or in vivo.

RAGE does not contribute to DC migration or DC:T cell conjugate stability

Because it has been noted that RAGE interacts with the adhesion receptor CD11b implicated in neutrophil trafficking, RAGE contributions to DC migratory capacity were addressed as a possible explanation for the reduced T cell priming capacity of RAGE−/− mice (Fig. 2). In the first approach, BMDCs from RAGE+/+ and RAGE−/− mice were labeled with CFSE, injected into the hind footpaws of recipient mice. The draining popliteal and inguinal lymph nodes were recovered 24 h later, and the number of CFSE-labeled migrating DCs was enumerated by flow cytometry. Lymph nodes from recipients contained similar numbers of migrating CFSE-labeled RAGE+/+ and RAGE−/− BMDCs, suggesting that RAGE on DCs was not required for migration of DCs from s.c. sites through the lymph to draining nodes (Fig. 7A). To directly examine DC migration in situ, mice were skin painted with the fluorescent probe TRITC which labels cutaneous DCs and permits evaluation of their migration to draining lymph nodes (20). After application of TRITC to the skin of RAGE+/+ and RAGE−/− mice similar numbers of migratory cutaneous DCs were found in the draining inguinal and brachial lymph nodes (Fig. 7B). Thus, RAGE expression in vivo is not required for migration of skin DCs to draining lymph nodes, discounting this possible contribution of RAGE in the adaptive response.

To address the possibility that RAGE interactions could effect DC:T cell adhesive events, RAGE+/+ and RAGE−/− OT-II T cells were assessed for their ability to form stable conjugates with DCs. At both 1 and 24 h of coculture, peptide-dependent conjugate formation was comparable in the presence or absence of RAGE (Fig. 7, C and D), arguing against the importance of RAGE-mediated adhesive events.

Discussion

We have identified RAGE as an activation receptor on T cells contributing to both the amplitude and the quality of T cell priming. RAGE-deficient T cells demonstrated impaired strength of proliferative responses in vitro and in vivo. These data are consistent with prior studies implicating RAGE in T cell activation that have used blocking Abs (12, 13) capable of blocking RAGE expressed by either T cells or APCs. The data presented here using adoptively transferred RAGE-deficient cells implicate intrinsic T cell RAGE signaling as an important modulator of T cell activation. In addition the effector outcome of responding T cells is influenced by T cell-expressed RAGE. Restimulated RAGE-deficient T cells were impaired in both IFN-γ- and IL-2 production, whereas elaboration of prototypical Th2 cytokines was enhanced. Thus, RAGE engagement on T cells may alter the balance of Th1/Th2 immunity. Impaired induction of effector T cell responses is not due to intrinsic developmental T cells defects or increased natural regulatory T cell populations in RAGE−/− mice, given that direct activation of RAGE−/− CD4+ T cells by anti-CD3 is not altered; nor do we find increased numbers of FoxP3+ CD4+ CD25+ T cells in the lymph nodes and spleens of RAGE−/− mice (data not shown).

We find that RAGE expression increases during initial T cell activation, being expressed at low levels in resting naive T cells and more prominently by memory T cells. Consistent with these expression patterns, Dimitriu et al. (13) found that HMGB-1 stimulated preactivated T cells but not naive resting T cells. The prediction of these expression kinetics, which remains to be assessed, is that RAGE signaling on T cells would contribute during later stages of initial T cell activation and to restimulation of memory.

RAGE expressed by non-T cells was also found to contribute to T cell priming as proliferative responses were also inhibited after transfer of RAGE+/+ OT-II cells into hosts which lacked RAGE expression. We predicted that the most likely RAGE-expressing cells responsible for the host defect would be DCs. However, in vivo transfer of peptide-loaded RAGE-deficient DCs led to normal OT-II-proliferative responses in vivo (Fig. 5A). These findings were surprising because recent studies have noted that the RAGE ligand HMGB-1 is released from DCs and can contribute to DC
maturation and function (21) after RAGE and/or TLR2, -4, and -9 engagement (13, 19, 22–24). However, the data reported here reveal that RAGE-/- BMDCs and splenic DCs responded normally to TLR stimulation, up-regulating surface expression of both CD86 and MHC class II and secreting normal levels of immunostimulatory cytokines. Furthermore, activation of OTZ-I and OTZ-II hybrids, which are responsive simply to quantitatitive peptide:MHC loading, is also not affected by the absence of RAGE on DCs. Thus, DC RAGE signaling is not required for antigenic internalization/processing or for TLR activation in vitro or in vivo.

Similar to T cells, RAGE on DCs was found to be up-regulated by cellular activation, although this was observed only after immune complex stimulation and not by either TLR or anti-CD40 activation. The induction of RAGE expression by immune complexes was inhibited in the presence of HMGB-1-blocking Abs consistent with the notion that RAGE expression may be positively regulated by its ligands.

The host defect observed in T cell priming after CFA/OVA immunization could instead have been due to impaired migratory capacity of Ag-loaded DCs. Indeed, it has been noted that RAGE interaction with integrins, including CD11b/CD18, are involved in neutrophil peritoneal migration (7). Furthermore, blocking Abs to the RAGE ligand HMGB-1 perturbs human migratory function in vitro (25, 26). However, RAGE-/- DCs migrated normally to draining lymph nodes from the skin or after transfer into footpads. These conflicting data regarding our studies with RAGE-/- DCs and previous reports documenting the DC inhibitory capacity of various RAGE antagonists (either sRAGE or blocking Abs to RAGE or the ligand HMGB-1) might be reconciled by the presence of additional HMGB-1 receptors expressed by DCs including TLR2 and -4 (27, 28).

Consistent with this notion, sRAGE- and anti-HMGB-1-blocking Abs inhibited Ag presentation by both RAGE+/- and RAGE-/- DCs (data not shown). Thus, the presence of multiple redundant HMGB-1 receptors on DCs may make RAGE dispensable for HMGB-1 signaling on DCs, whereas on T cells the lack of endogenous TLR expression renders RAGE essential for the T cell response to HMGB-1.

Thus, our data support an important role for RAGE on T cells in adaptive immunity. The impaired capacity of RAGE-/- recipient mice to support OT-II-proliferative responses appears not to be due to intrinsic DC defects in either Ag processing, activation or migratory capacities. Future studies will address whether RAGE expression on other immunologically relevant cells additionally contribute to adaptive responses in vivo.

The outcome of the cognate DC:T cell interaction, immunity vs tolerance, is influenced by the integration of multiple signaling inputs by T cells, in addition to the strength of TCR signaling. These additional activation signals include both cell surface molecules (adhesion and costimulatory B-7 family) and secreted molecules (e.g., cytokines, chemokines). These molecular mediators are provoked in tissue by inflammation-promoting T cell activation and immunity. Many molecular microbial adjuvants pivotal to cellular activation in infectious immunity have been characterized, including TLR ligands. However, sites of inflammation likely contain other endogenous, host-derived signaling systems, including HMGB-1 released by DCs and by bystander cells that can influence the DC:T interaction in the absence of microbial stimuli. Indeed recent studies have shown that commonly used immunological adjuvants, e.g., Freund’s adjuvant, enhance immunity at least in part in a TLR-independent manner (29), suggesting the existence of other responsible activation-signaling pathways. RAGE is a candidate activation receptor on T cells for regulation of the adaptive host immune response, because its ligands are inducibly present at sites of inflammation. We conclude that the RAGE signaling pathway may signal tissue injury, providing an endogenous activator of T cell effector immunity.

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Disclosures

The authors have no financial conflict of interest.

References


