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Complement C3a, CpG Oligos, and DNA/C3a Complex Stimulate IFN-α Production in a Receptor for Advanced Glycation End Product-Dependent Manner

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The receptor for advanced glycation end products (RAGE) is a multiligand transmembrane receptor implicated in a number of diseases including autoimmune diseases. To further understand the pathogenic mechanism of RAGE in these diseases, we searched for additional ligands. We discovered that C3a bound to RAGE with an EC_{50} of 1.9 nM in an ELISA, and the binding was increased both in magnitude (by >2-fold) and in affinity (EC_{50} 70 pM) in the presence of human stimulatory unmethylated cytosine-guanine-rich DNA A (hCpGAs). Surface plasmon resonance and fluorescence anisotropy analyses demonstrated that hCpGAs could bind directly to RAGE and C3a and form a ternary complex. In human PBMCs, C3a increased IFN-α production in response to low levels of hCpGAs, and this synergy was blocked by soluble RAGE or by an Ab directed against RAGE. IFN-α production was reduced in response to mouse CpGAs and C3a in RAGE^{-/-} mouse bone marrow cells compared wild-type mice. Taken together, these data demonstrate that RAGE is a receptor for C3a and CpGA. Through direct interaction, C3a and CpGA synergize to increase IFN-α production in a RAGE-dependent manner and stimulate an innate immune response. These findings indicate a potential role of RAGE in autoimmune diseases that show accumulation of immunostimulatory DNA and C3a.

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As a pattern recognition receptor, a receptor for advanced glycation end products (RAGE) is known to bind to danger-associated molecular patterns (DAMPs) that are released by cells that are dying or under inflammatory conditions. Characterized RAGE ligands include S100 proteins (1), HMGB1 (2), advanced glycation end products (AGEs) (3), the cell adhesion receptor MAC-1 (4), and amyloid-β peptide (5). Expression of RAGE is increased at sites of inflammation (6), and soluble RAGE protein is protective in the CLP model of sepsis, collagen-induced arthritis, and the experimental allergic encephalitis model of multiple sclerosis (7, 8). RAGE^{-/-} mice are resistant in the CLP model of sepsis, and blocking Abs are protective. RAGE has been demonstrated to mediate inflammation in response to DAMPs and has been hypothesized to play an important role in acute and chronic inflammatory diseases.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease, characterized by autoantibodies and systemic clinical manifestations (9). Abnormalities in apoptosis lead to the release of DAMPs and nuclear components that stimulate the immune system to produce autoantibodies including anti-DNA Abs, activate the complement system, and damage tissues (10–15). This process initiates a pathogenic cycle in which both the adaptive and the innate immune system collaborate to advance the disease. In addition to the hallmark anti-nuclear and other autoantibodies, serum levels of high-mobility group box protein (HMGB)1 (16), C3a (17), IFN-α (18), and dsDNA (19) are consistently high in patients with lupus and correlate with the disease severity.

Investigating the molecular mechanisms of how the innate immune system is involved in the pathogenesis of lupus is of intense interest. IFN-α production induced by autoantibody complexes containing immunostimulatory nucleic acids is hypothesized to have a major role in the pathogenesis of SLE in a TLR9- and TLR7-dependent manner (18). For example, DNA-containing immune complexes have been shown to bind to the FcγRIIA receptor (20) expressed on plasmacytoid dendritic cells (pDCs), DNA is endocytosed to activate TLR7/9 pathways and increase IFN-α production. The IFN-α produced by the innate immune system in this manner then stimulates a strong autoimmune response from the adaptive immune system by activating T cells and inducing more autoantibody production by B cells (21).

HMGB1, a nuclear transcription factor that binds DNA with high affinity (22), is seen released from cells under inflammatory conditions and bound to DNA in SLE serum (23). The function of extracellular HMGB1 as a DAMP has attracted great attention. Binding of HMGB1 has been demonstrated to RAGE, TLR2, and TLR4, and each of these receptors has been demonstrated to...
Limitus amebocyte lysate method) were used in cell-based assay. XTH2 chased from Sigma-Aldrich (St. Louis, MO) immunized with an hRAGE expression plasmid and boosted with hRAGE associated with inflammation or danger. The Costar3590 assay plates were coated with IFN-γ. An ELISA was established to screen for hRAGE binding to proteins associated with inflammation or danger. The Costar3590 assay plates were coated with IFN-γ. An ELISA was established to screen for RAGE ligands by ELISA to screen for RAGE ligands. ELISA to screen for RAGE ligands.

Materials and Methods

Materials

Cpg oligonucleotides were purchased from InvivoGen (San Diego, CA) and used after reconstitution, according to the manufacturer’s instructions. Where stated, the Cpg oligos were heat treated by brief heating in a boiling water bath and cooled prior to use in multiangular dynamic light scattering experiments and in the Biacore (Piscataway, NJ). Human and mouse RAGE-Fc (hRAGE and mRAGE) and Fc control proteins and anti-RAGE Abs were made by the Pfizer Biological Technologies Group (Cambridge, MA) by standard methods and are described elsewhere (26). S100A4, C3a, C5a, C1r, and HMGB1 were purchased from R&D Systems (Minneapolis, MN), and C3a and C5a were from Calbiochem (San Diego, CA). S100A5, S100A6, S100A7, S100A8, and S100A9, and secreted protein acidic and rich in cysteine (SPARC) were from Novus Biologicals (Littleton, CO). Heat shock protein (Hsp)60, Hsp70, and Hsp73 were purchased from StressGen Biotechnologies (Ann Arbor, MI). Fibrinogen fragment E, fibrinogen fragment D, and fibrinogen were purchased from Haemostatics Technologies (Essen Junction, VT). C3a, HMGB1, and C3adesArg (=95% pure with endotoxin level<1.0 Eu/1 µg protein by the Limulus amebocyte lysate method) were used in cell-based assay. XTH2 and XTH5 were isolated by screening hybridomas derived from mice immunized with an hRAGE expression plasmid and boosted with hRAGE protein (26). Other reagents, including tissue culture media, were purchased from Sigma-Aldrich (St. Louis, MO).

ELISA to screen for RAGE ligands

An ELISA was established to screen for RAGE binding to proteins associated with inflammation or danger. The Costar 3590 assay plates were coated with protein ligands (2 µg/ml) for overnight in PBS at 4˚C, washed in washing buffer (PBS with 0.05% Tween 20) at 25˚C, and blocked for 2 h in blocking buffer (phosphate buffered saline with 0.05% Tween 20 and 1% BSA) at 25˚C. To the ligand-coated plates, hRAGE protein (0, 1, 3, and 10 µg/ml) or control proteins (0, 10, 30, and 100 µg/ml) or competing Abs were added in blocking buffer, incubated for 1 h at 25˚C, washed with washing buffer, and treated with anti-human IgG HRP (1/10,000 in blocking buffer; Jackson ImmunoResearch Laboratories, West Grove, PA) to reveal the binding to the ligands.

The synergistic effect of Cpg oligos in RAGE binding to its ligands (e.g., C3a) was measured by incubating ligand (e.g., C3a)-coated plates with hRAGE (or Fc control) in the presence of 0.3 µCpg oligos (e.g., human stimulatory CpG-hCpGAs, mouse CpGAs [mCpGAs], hCpGAc, and human stimulatory CpG Bs [hCpGBs]).

To test the function of anti-RAGE Abs and mRAGE in blocking C3a binding to RAGE in the presence and absence of hCpGAs, a C3a-coated ELISA plate was incubated with a mixture of hRAGE (0.03 µg/ml) and anti-RAGE Abs (or mRAGE) in the presence or absence of hCpGAs (0.3 µM) in blocking buffer.

Fluorescence anisotropy analysis to measure direct binding of hRAGE to FITC-hCpGAs

FITC-labeled hCpGAs (3 nM) were mixed with series dilutions of hRAGE or Fc in the PBS-Tween buffer in duplicate in black 96-well, polystyrene, flat-bottomed plates for 15 min to reach equilibrium. Fluorescence anisotropy at the steady state was measured by the Analyst AD spectrofluorometer plate reader (LJL, Biosystems, Molecular Devices, Sunnyvale, CA) using filters (excitation = 485 nm, bandpass = 20 nm; emission = 505 nm, bandpass = 25 nm), dichroic mirror = 505 nm, and the anisotropy values were calculated using the relationship \( r = I_{45} - I_{135} / I_{45} + I_{135} \), where the difference in intensity is normalized by the total intensity.

Biacore analysis of direct binding of RAGE to Cpg oligos

hRAGE, Fc, HMGB1, and C3a proteins were immobilized on a CMS Biacore sensor chip surface by amine coupling (Biacore, NJ) in BIA-buffer containing 20 mM Hepes (pH 7.5), 150 mM NaCl, 2 mM MgCl2, and 0.008% Triton X-100. Briefly, the CMS Biacore chip was activated with a mixture of N-ethyl-N-(2-dimethylaminopropyl)carbodiimide hydrochloride and N-hydroxysuccinimide, hRAGE (20 µg/ml in 10 mM sodium acetate [pH 5.5]) or Fc (20 µg/ml in 10 mM sodium acetate [pH 5]) directly captured through amine coupling, and deactivated with 1 M ethanolamine-HCl. Two different density surfaces were prepared for each of the following proteins, hRAGE (12,000 or 2.5 × 10^11 response units [Ra]), Fc (8,159 or 1,200 Ru), HMGB1 (4,800 or 1,000 Ru) and C3a (3,000 or 500 Ru). The amine coupling blocked surface was used as the blank control flow cell. Real-time biomolecular interaction between heat-treated Cpg oligonucleotides, and hRAGE protein were performed on a Biacore 3000 surface plasmon resonance instrument using BIA buffer with 0.02% BSA at 25˚C. A series of 2-fold dilutions of hCpGAs, hCpGAc, hCpGBs, hCpGAc, and CpGcIgant from 0 to 2000 nM were injected at 25˚C. 30 µl/min. All surfaces were completely regenerated by a 30-s pulse of a solution of 550 mM MgCl2, 140 mM potassium thiocyanate, 280 mM urea, and 550 mM guanidine-HCl, followed by two consecutive 30 s. injections of 10 mM Glycine (pH 1.5) buffer. All experiments were done in duplicate, and the data were processed using the blank surface as reference and buffer injections as background.

Biacore analysis of complex formation among RAGE, C3a, and CpgAs

The assay was performed using the BIA-buffer with 0.02% BSA as described above. Heat treated Cpg oligos (100 nM) were injected on a C3a sensor chip surface followed by a series of 2-fold dilutions of RAGE (0–20 µg/ml) using the coinject command. In separate experiments, Cpg oligos were injected on a RAGE sensor chip surface, followed by HMGB1 or C3a using the coinject command. In competition experiments, solutions of RAGE (2.5 µg/ml) plus dilutions of heat-treated CpgAs (0–1000 nM; 2-fold) were injected on a sensor chip surface composed of a complex of C3a and CpgAs. The complex of C3a and CpgAs was formed by injecting CpgAs (1 µM) to the C3a immobilized surface. The binding signals for RAGE were calculated by subtracting the background obtained from the buffer injections.

Size exclusion chromatography and multiangular light scattering analysis of immunostimulatory Cpg oligos

Cpg oligos were freshly dissolved in deionized water at 50 µM concentration, and aliquots were taken for treatment at 95˚C for 10 min and then at 4˚C for 20 min. For analysis of multimerization, the Cpg oligos (25 µg) were injected onto an YMC-Pack Diol-300 column (500 × 8.0 mm ID; Waters, Milford, MA) installed in a Waters HPLC unit, and the column was developed in PBS buffer at a flow rate of 1 ml/min. The elution of Cpg oligos was detected by a Mini Dawn TriSTAR multiangular light scattering device connected in tandem to an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA). The homogeneity and m.w. of the Cpg oligos were determined by size exclusion chromatography-multiangular light scattering (SEC-MALS) analysis.

Human PBMC-based assay

Leukopacks were obtained from Research Blood Components (Boston, MA). PBMCs were isolated by Histopaque 1077 (H8889; Sigma-Aldrich) density gradient centrifugation as described previously. After washing, the PBMCs were suspended in RPMI 1640/5% FBS and plated at 2000 cells/well (100 µl) in a 96-well tissue culture plate (3072; BD Falcon, Franklin, NJ). To the plate, mixtures of proteins (endotoxin free) and hCpGAs were added to give a final total volume of 200 µl and incubated for 20 h. The supernatants from the assay were collected for IFN-α...
measurement by ELISA (Bender MedSystems, Vienna, Austria). Cell viability was measured using cell proliferation reagent WST-1 (Roche, Basel, Switzerland).

**Human pDC assays**

Human pDCs were isolated from PBMCs by magnetic bead separation using the Miltenyi BDCA4 (CD304) direct isolation kit (order number 130-090-532; Miltenyi Biotec, Auburn, CA) and stimulated for overnight at 40,000 cells/well concentration in 0.2 ml complete media (RPMI 1640/10% FBS supplemented with l-glutamine, nonessential amino acids, sodium pyruvate, penicillin, streptomycin, and 2-ME). The supernatants from each assay were collected for IFN-α measurement using the Bender MedSystems ELISA kit.

**Mouse bone marrow and pDC assays**

The long bones were harvested from RAGE−/− mice (SVJ129) (26) or age- and sex-matched wild type controls. The total bone marrow was removed by lavage using a 21-gauge hypodermic needle, and the RBCs were removed by treatment with EasySep RBC lysis buffer (StemCell Technologies). After washing, the cells were suspended in RPMI 1640/5% FBS and plated at 200,000/well in a 96-well plate and treated as described above for human pDCs. After 20-h incubations, the supernatants were harvested for IFN-α measurement by ELISA (PBL IFN source). Cell viability was measured using cell proliferation reagent WST-1 (Roche). The mouse pDCs were isolated using the mouse pDC isolation kit (order number 130-091-263; Miltenyi Biotec), and 40,000 cells/well were treated as described above for human pDCs, except that mCpGAs and mRAGE were used.

**Results**

**C3a, HSP70, and SPARC are novel RAGE-binding proteins**

To identify novel RAGE ligands and compare the affinities to known RAGE ligands, we tested 28 DAMP proteins by a functional ELISA. HMGB1 and several S100 proteins are known ligands for RAGE. However, not all of the tested S100 proteins bound to RAGE under our experimental conditions, and their binding affinities varied from strong to no binding as shown in Table I. Interestingly, we discovered novel RAGE binders, such as HSP70, SPARC, and complement component C3a and C3adesArg. The high-affinity binding to the lupus-related molecule C3a appears to be selective, and other complement molecules such as the structurally related C5a peptide, C4, and C1R did not bind to RAGE (Fig. 1, Table I). Taken together, among the 28 DAMP proteins tested, C3a and C3adesArg appear to be a high-affinity binders to RAGE.

**CpG oligonucleotides bind directly to RAGE, C3a, and HMGB1**

CpG oligos are sequence specific immunostimulatory molecules that differ in physical and biological properties as shown in Table II. The mCpGAs was reported to form a complex with HMGB1 (2), and is a pathogen-associated molecular pattern mimicking bacterial unmethylated DNA, so we tested its binding to RAGE and C3a. Both the fluorescence anisotropy analysis (Fig. 2A) and Biacore analysis (Fig. 2C) showed that hRAGE bound directly to hCpGAs (Table II) with high affinity. The apparent Kd for hCpGAs binding to hRAGE in the fluorescence anisotropy assay was 20 nM. In Biacore analysis, saturable binding to hRAGE was reached at 1 μM hCpGAs. However, the binding is complex, indicated by lack of fit of the data from the corresponding sensorgrams to a 1/1 model, perhaps as a result of the multimeric nature of hCpGAs (Table II) (27). For this reason we used steady-state analysis from Fig. 2A to calculate an apparent Kd of 34 nM for hCpGAs binding to hRAGE.

We further evaluated the binding of various CpG oligos to hRAGE, hFc, HMGB1, and hC3a proteins. Because we could not obtain a precise kinetic data (Kon and Koff) by fitting the curves with a 1/1 model, we compared the sensorgrams at a Yes-or-no binding level. Similar binding patterns on either high-density (Fig. 2B–E) or low-density chips (data not shown) were observed. There was no binding of any of the four CpG oligonucleotides to the Fc control protein, indicating the absence of non-specific binding (Fig. 2B). In contrast, hCpGAs bound to hRAGE (Fig. 2C) with an apparent slow dissociation rate. The hCpGAc, in which the CpG motifs of hCpGAs are inverted to GpC, showed almost no binding to hRAGE. The stimulatory hCpGBs and the inhibitory mCpGi also each bound to hRAGE. pCpG giant had no binding (data not shown) to hRAGE.

hHMGB1 (Fig. 2D) and hC3a (Fig. 2E) each bound to the CpGs in a similar pattern. They bound hCpGAs with an apparent Kd calculated from steady state binding of 12 and 20 nM for HMGB1 and C3a, respectively. Saturable binding was reached at 62 and 100 nM hCpGAs for HMGB1 and C3a, respectively. When more hCpGAs (>100 nM) was injected, dose-dependent reduction of hCpGAs binding to HMGB1 or C3a was observed. In addition, hCpGBs and mCpGi bound hHMGB1 and hC3a, but hCpGAc or pCpG giant showed only very weak or essentially no binding. Taken together, RAGE, C3a, and HMGB1 bind to CpG containing oligos (hCpGAs, hCpGBs, and mCpGi) but do not bind to non-CpG-containing controls (e.g. pCpG giant and hCpGAc), suggesting a role for the CpG motif in binding. Also, the trimeric hCpGAs showed ~3-fold higher RAGE-binding signal than the hCpGB and mCpGi, indicating that the binding is influenced by DNA sequence and perhaps also by their ability to self associate to form multimers.

To further understand the nature of the CpG oligos, we measured its molecular size by SEC-MALS. As shown in Table II, CpG oligos freshly reconstituted from lyophilized powder formed high order

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Table I. Binding of DAMPs to sRAGE-Fc

<table>
<thead>
<tr>
<th>RAGE Ligands</th>
<th>Binding Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGB1</td>
<td>+++</td>
</tr>
<tr>
<td>S100P</td>
<td>–</td>
</tr>
<tr>
<td>S100AA h</td>
<td>+++</td>
</tr>
<tr>
<td>S100B</td>
<td>–</td>
</tr>
<tr>
<td>S100A5</td>
<td>++</td>
</tr>
<tr>
<td>S100A6</td>
<td>++</td>
</tr>
<tr>
<td>S100A7</td>
<td>+</td>
</tr>
<tr>
<td>S100A8</td>
<td>+</td>
</tr>
<tr>
<td>S100A9</td>
<td>+</td>
</tr>
<tr>
<td>Potential Ligands</td>
<td></td>
</tr>
<tr>
<td>APOa1</td>
<td>–</td>
</tr>
<tr>
<td>APOa2</td>
<td>–</td>
</tr>
<tr>
<td>MIF</td>
<td>–</td>
</tr>
<tr>
<td>Gelsolin</td>
<td>nonspecific</td>
</tr>
<tr>
<td>C3a</td>
<td>+++</td>
</tr>
<tr>
<td>Complement C3</td>
<td>+</td>
</tr>
<tr>
<td>Complement C4</td>
<td>nonspecific</td>
</tr>
<tr>
<td>C3ades/Arg</td>
<td>+++</td>
</tr>
<tr>
<td>C5a</td>
<td>–</td>
</tr>
<tr>
<td>C1R</td>
<td>nonspecific</td>
</tr>
<tr>
<td>HSP60</td>
<td>+</td>
</tr>
<tr>
<td>HSP70</td>
<td>++</td>
</tr>
<tr>
<td>HSP73 bovine</td>
<td>+</td>
</tr>
<tr>
<td>HSP1650</td>
<td>nonspecific</td>
</tr>
<tr>
<td>SPARC</td>
<td>++</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>+</td>
</tr>
<tr>
<td>Fibrinogen FE</td>
<td>–</td>
</tr>
<tr>
<td>Fibrinogen FD</td>
<td>+</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>–</td>
</tr>
</tbody>
</table>

*Binding of DAMPs to hRAGE. DAMPs were coated on ELISA plates and tested for binding to hRAGE (0, 1, 3, and 10 mg/ml) or Fc control (0, 10, 30, and 100 mg/ml).

+++ Strongest specific binding at the lowest concentration of hRAGE; ++, strong binding; +, weaker binding; +, very weak binding observed only at the highest concentration; nonspecific, binding to the Fc control.
complexes that varied from monomer, dimer, trimer, or multimers in a sequence-dependent manner. After heat treatment, the reconstituted CpG oligos were less aggregated. The mCpGi changed from a multimer (27 kDa) to a monomer (6 kDa; expected monomer is 5 kDa). hCpGAs changed from a large complex to a trimer, and the mCpGAs remained a large complex after heat treatment. The hCpGBs remains a monomer (9 kDa; expected monomer is 7.7 kDa) both before and after heat treatment.

The lack of pure monomers in the CpG preparations explains the difficult to fit curves from Biacore sensorgrams and suggests that the avidity increase derived from having multiple binding sites plays a strong role in the behavior of the interaction of CpG oligos, RAGE, and C3a. The biological and biochemical competition studies in this paper were done with oligos freshly reconstituted according to the manufactures instructions unless otherwise stated.

RAGE, CpgA and C3a can form ternary complexes

We have shown that the hCpGAs binds directly to hRAGE, hC3a and hHMGB1 through its CpG motif (Fig. 2). hCpGAs contains three CpG motifs in its DNA sequence and can form large aggregates in solution (Table II). We tested to see whether CpGAs could bind to different proteins (e.g., RAGE and C3a) simultaneously through the CpG motifs of a multimer and in this way mediate a ternary complex between RAGE and C3a.

We immobilized the first analyte on a Biacore CM5 sensor chip, and then used the cojunction method where an injection of the second analyte is followed immediately the third analyte. If the second analyte binds to the first analyte, and the third analyte can bind to the complex of the first and second analytes, we will expect to see two-step increase in the binding signal. The absence of a diminished signal in the second step would suggest that the second and third analytes compete for binding to the first analyte. The hCpGAs (100 nM) bound to the C3a sensor chip surface and reached a steady state signal of ~450 Ru (Fig. 3A). hRAGE (1–20 μg/ml) bound to the C3a/CpGAs surface in a dose-dependent manner. This clearly demonstrates the formation of a ternary complex among C3a, hCpGAs and hRAGE. To confirm this finding, we reversed the experiment, immobilizing hRAGE on the CM5 chip, and coinjecting hCpGAs, followed by C3a (Fig. 3B). These data show that ternary complex formation among hRAGE, hCpGAs, and C3a is not dependent on the order of injection.

We then tested the ternary complex formation using mRAGE, mCpGAs, and mCpGi, which contain only 1 CpG motif in their DNA sequences (Table II). On the basis of SEC-MALS analysis, mCpGi is a monomeric-like after heat treatment, whereas mCpGAs is a large multimeric complex containing several CpG motifs (Table II). Interestingly, ternary complex formation among C3a–mCpGAs–mRAGE was observed when either hC3a (Fig. 3C) or mRAGE (Fig. 3D) were immobilized on the sensor chip. In contrast, although the monomeric mCpGi (after heat treatment) could bind directly to C3a surface and reached a steady state binding of 400 Ru at the end of injection, no ternary complex was seen with hRAGE. Instead, when the second injection of hRAGE was made, we observed dose-dependent decrease of the binding signal immediately after the injection, followed by rebinding of hRAGE to the refreshed C3a surface (Fig. 3E). These data show that the monomeric mCpgi could not form a ternary complex and that hRAGE directly competed with mCpGi for binding to C3a. Similarly, no ternary complex with hRAGE and C3a were observed, when the monomeric-like CpGBs was used (data not presented). Taken together, the ternary complex formation among hRAGE, C3a and CpG oligos may be dependent on the availability of multiple CpG motifs either from the DNA oligos or by the formation of oligo multimers.

CpGAs serves as a linker in ternary complex formation among RAGE, CpGAs, and C3a

The multiple CpG requirement for ternary complex formation suggested that CpGAs may serve as a linker between RAGE and C3a. If this is the case, excess amount of CpGAs should prevent RAGE.

Table II. CpG oligonucleotides form multimers

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Oligo Specificity</th>
<th>ssDNA Sequencea</th>
<th>CpG Motib</th>
<th>Base Pairc</th>
<th>SEC-MAL Analysis Untreatedd</th>
<th>SEC-MAL Analysis Heat-Treatedd</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODN2216</td>
<td>Human-specific stimulatory hCpGAs</td>
<td>5'-ggGGGACACTGTCggcggg3-3'</td>
<td>Yes 10</td>
<td>Large complex</td>
<td>17 kDa (trimer, 99.7%)</td>
<td></td>
</tr>
<tr>
<td>ODN2216 control</td>
<td>Nonstimulatory hCpGAc</td>
<td>5'-ggGGGACACTGTCgcgg gc3-3'</td>
<td>No 8</td>
<td>8 kDa (61%) 13 kDa (2%) 18 kDa (40%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ODN1585</td>
<td>Mouse-specific stimulatory mCpGAs</td>
<td>5'-gggGTCACGTGAgcggg3-3'</td>
<td>Yes 10</td>
<td>Large complex</td>
<td>Large complex</td>
<td></td>
</tr>
<tr>
<td>ODN2006</td>
<td>Human-specific stimulatory hCpGBs</td>
<td>5'-tcggcggcccgttggtgt3-3'</td>
<td>Yes 9</td>
<td>9 kDa (monomer, 99.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ODN2088</td>
<td>Mouse-preferred inhibitory CpGa mCpGai</td>
<td>5'-tcggcggggga3-3'</td>
<td>Yes 27 kDa (62%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCpG giant</td>
<td>Nonstimulatory</td>
<td>CpG-free giant plasmid</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aDNA bases shown in capital letters are phosphodiester, and those in lowercase are phosphorothioate (nuclease resistant). Palindrome is underlined. Bases shown in bold can be used to form dsDNA.
bThe presence of CpG motifs.
cNumber of the bases shown in bold can be used to form dsDNA.
dMolecular weight (grams per milliliter) with 10% error. FW, formula weight.
from binding to CpGA–C3a complex. To test this hypothesis, we immobilized the hC3a/hCpGAs complex on the chip surface first, and then injected a mixture of hRAGE (2.5 μg/ml) and increasing amounts of hCpGAs (0–1000 nM). If hCpGAs and hCpGAs/hC3a complexes compete for the same binding site on hRAGE, a high concentration of hCpGAs will result in a lower concentration of free RAGE available for binding to the hC3a/hCpGAs complex on the chip surface. We observed that at low hCpGAs concentration (0–30 nM), there was a steady-state RAGE-binding signal of 2000 Ru (Fig. 3F). As the hCpGAs concentration was increased (60–1000 nM), less free hRAGE was available for binding, and the steady-state hRAGE-binding signal decreased in a dose-dependent manner. The competition seen with the soluble hCpGAs demonstrated that hCpGAs can mediate ternary complex formation.

Ternary complex formation among RAGE, CpGAs, and HMGB1 is conditional

We tested the ability of RAGE, CpGAs and HMGB1 to form a ternary complex. HMGB1 was immobilized on the CM5 surface, and then hCpGAs or mCpGAs were injected to make HMGB1/CpGAs complex, followed by injecting of RAGE proteins. Both mRAGE and hRAGE (2.5–20 μg/ml) showed binding to the HMGB1/CpGAs complex, indicating a possible ternary complex formation (Fig. 4A, 4B).

However, when the experiment is reversed as such that RAGE was immobilized on the CM5 chip and CpGAs was injected to form a RAGE/CpGAs complex followed by HMGB1 injection, HMGB1 (4–30 μg/ml) injections did not show additional binding (Fig. 4C, 4D). In fact, when HMGB1 was injected on to the hRAGE/hCpGAs complexed surface, we observed a decrease in the binding signal immediately after the injection, suggesting that the high-affinity binding of HMGB1 to hCpGAs prevented rebinding of hCpGAs to hRAGE, and there was no stable ternary complex formation. Similar results were obtained using mRAGE and mCpGAs, although less mCpGAs was removed from mRAGE by HMGB1.

These results show that ternary complex formation among RAGE–CpGAs–HMGB1 is conditional, perhaps depending on the relative affinities among RAGE, HMGB1, and DNA, the ratios of these components, and the availability of free CpG motifs for additional binding. Potentially the conditional nature of ternary complex formation could contribute to the variation in biological activity of HMGB1; HMGB1 has been reported to increase mCpGAs induced IFN-α production in mouse but to reduce the hCpGAs effect in human (2, 23, 25).

H5 anti-RAGE Ab and mRAGE prevent hRAGE from binding to C3a in the presence of hCpGAs

In a functional ELISA, hRAGE bound to C3a-coated plates with an EC₅₀ of 1.9 nM (Fig. 5A). In the presence of hCpGAs, the maximal binding of RAGE to C3a-coated plates increased by >2-fold, and the EC₅₀ value decreased to 70 pM indicating higher binding.
affinity (Fig. 5A). This synergistic effect mediated by hCpGAs is consistent with results from the Biacore-based assay, which showed that hRAGE could bind directly to hC3a or to the hCpGAs/hC3a complex (Figs. 2A, 2C, 3A, 3B). In addition, C3adesArg also binds to hRAGE with an EC50 of 30 nM. In the presence of hCpGAs, the maximal binding of RAGE to C3adesArg-coated plates increased by 2-fold, and the EC50 value decreased to 17 nM, indicating higher binding affinity (Fig. 5C). Interestingly, this preparation of C3ades-Arg is known not to bind to the C3a receptor and yet has high-affinity interaction with RAGE.

To find an agent that blocks hRAGE from binding to C3a in the presence of hCpGAs, we tested anti-RAGE Abs (XTH2 and XTH5).
and soluble mRAGE (26). Both Abs blocked HMGB binding to hRAGE (data not shown). Interestingly, H5 but not H2 is able to completely block hRAGE binding to the hC3a/hCpGAs complex (Fig. 5B). As expected, an mRAGE is also an effective competitor, and the control anti- Eimeria tenella Fc (an IgG1 isotype control) has no effect (Fig. 5B). Both anti-RAGE H5 Ab and mRAGE provided valuable tools for analysis of the biological effects of the hRAGE/hCpGAs/hC3a interactions.

RAGE mediates mCpGAs/C3a-induced IFN-α production in mouse pDCs and bone marrow cells

We next measured mCpGAs induced IFN-α production in mouse pDCs in the presence of different amounts of mRAGE or IgG protein. As shown in Fig. 6A, although the control IgG2a did not reduce 0.3 μg/ml mCpGAs induced IFN-α production, mRAGE (1–10 μg/ml) blocked IFN-α production in a dose-dependent manner, showing that mRAGE could block 0.3 μg/ml mCpGAs induced IFN-α production.

The biological effects of mCpGAs, C3a and/or HMGB1 were measured through IFN-α production in mouse pDCs isolated from mouse bone marrow. In the absence of mCpGAs, no detectable amount of IFN-α was produced in the presence of C3a, HMGB1 (Fig. 6B) or lps (data not presented). To see a dramatic difference between C3a or HMGB1-treated bone marrow cells (BMCs) and untreated BMCs in the presence of mCpGAs, a suboptimal amount of 0.04 μg/ml mCpGAs was used where little IFN-α can be detected. Addition of HMGB1 or C3a greatly enhanced IFN-α production (Fig. 6B), indicating mCpGAs works synergistically with both C3a and HMGB1 in IFN-α production.

Because C3a is a ligand for C3aR and other receptors besides RAGEs, we wanted to test whether the synergistic effect of mCpGAs and C3a in IFN-α production was RAGE dependent. We isolated total BMCs from RAGE−/− mice and corresponding RAGE+/+ control mice. At lower concentrations of 0.3 μg/ml mCpGAs, a small amount of IFN-α production (20 pg/ml) was observed in RAGE−/− strain in response to mCpGAs, indicating there are RAGE-independent pathways mediating mCpGAs induced IFN-α production. Under the same conditions, 4-fold more IFN-α (80 pg/ml) was produced in RAGE+/+ strain, indicating the RAGE-dependent pathway is more efficient in mediating mCpGAs induced IFN-α production. Interestingly, in RAGE−/− strain, treatment with a mixture of 0.3 μg/ml mCpGAs and C3a generated strong IFN-α production (250 pg/ml) which is 10-fold higher than that RAGE−/− strain (25 pg/ml IFN-α), showing that RAGE is important in mediating synergistic effect of mCpGAs and C3a.

At higher concentrations of mCpGAs, no differences (data not shown) were seen in the maximal IFN-α production between RAGE−/− and RAGE+/+ mice, indicating that at high CpG concentrations IFN-α responses are RAGE independent; these RAGE-independent pathways for immune stimulatory DNA uptake might have lower affinity for DNA and become important at high DNA concentration. Taken together, the data demonstrate that C3a and CpGA synergize to produce IFN-α in a RAGE-dependent pathway.
and suggest that the C3a-CpGAs-RAGE ternary complex may be particularly important at low levels of CpG stimulation.

**C3a and hCpGAs synergize to produce IFN-α in a RAGE-dependent manner in human pDCs and PBMCs**

We tested whether the synergy between mCpGAs and C3a that we observed in the mouse would translate to a human cellular system. HMGB1 has been reported to increase mCpGAs-induced IFN-α production in mouse (2) but to reduce hCpGAs-induced IFN-α production in human cells (25). Under similar conditions, we also found that hHMGB1 inhibited hCpGAs-induced IFN-α production in primary human pDCs. In addition, we saw synergy in IFN-α production between mCpGAs and HMGB in mouse pDCs (Figs. 6B, 7A). These data are consistent with the Biacore-based binding results that showed that HMGB1 has high affinity for hCpGAs and efficiently competed against hRAGE for hCpGAs binding. Perhaps, in the presence of excess amount of HMGB1, much less hCpGAs can be taken up through a RAGE-dependent pathway.

Interestingly, hCpGAs and C3a showed a synergy in IFN-α production in both human PBMCs and mouse BMCs. As shown in Fig. 7B, in the absence of hCpGAs, a high concentration of C3a (10 μg/ml) did not induce IFN-α production, indicating that the potential interaction of C3a and surface C3aR or RAGE do not induce IFN-α production. However, in the presence of hCpGAs (2.5 μg/ml, 0.5 μM), C3a increased IFN-α production by several orders of magnitude. This synergistic effect perhaps is the result of efficient uptake of C3a and hCpGAs through ternary complex formation with hRAGE (or C3aR) and the delivery of the hCpGAs to TLR9 (2).

To further evaluate whether the synergistic effect is mediated by RAGE-dependent or RAGE-independent pathways (e.g., C3aR), we measured the effect of anti-RAGE Ab XTH5 in C3a/hCpGAs-induced IFN-α production (Fig. 7C). As shown in Fig. 5B, XTH5 could completely block RAGE from binding to C3a/hCpGAs complex, XTH2 was a partial blocker only at very high concentrations, and anti-*E. tenella* control IgG had no effect. Similar effects were also observed in human PBMCs in the presence of 5 μg/ml C3a and 2.5 μg/ml hCpGAs (Fig. 7C); XTH5 completely blocked C3a/hCpGAs induced IFN-α production, indicating that the synergistic effect of C3a/hCpGAs in IFN-α production is RAGE dependent. However, when the concentration of hCpGAs increased to 5 μg/ml, XTH5 partially blocked C3a/hCpGAs-induced IFN-α production, suggesting that in human as well as in mouse at high DNA concentration RAGE-independent pathways could play a role in C3a and hCpGAs induced IFN-α production. Interestingly, XTH2, an Ab that binds hRAGE with similar affinity to XTH5 and blocks HMGB binding to RAGE (data not shown) but does not block C3a/hCpGAs binding to hRAGE at the concentration used has no effect on IFN-α production. These data show exquisite epitope specificity that correlates between the biochemical assays and the functional cell biological assays and suggest a strong role for RAGE in the synergy between hCpGAs and C3a.

**Discussion**

RAGE is a pattern recognition receptor that not only binds to several S100 proteins, HMGB1, AGE, MAC-1, and amyloid-β peptide (16–18, 28), but also as we show in this paper, RAGE binds to HSP70, matricellular injury-related glycoprotein SPARC, complement component C3a, and CpG DNA oligos. These alarmin molecules are known to mediate various inflammatory diseases. DNA oligos, HMGB1, C3a are major pathogenic molecules in lupus (9). Lupus patients show serological accumulation of DNA, which through binding to TLR9 activates IFN-α production and drives a strong innate immune response (20, 21).

As a pattern recognition receptor, TLR9 binds to bacterial DNA and viral DNA, but not to the methylated genomic DNA from normal mammalian cells. Interestingly, TLR9 can be activated by DNA released from apoptotic and necrotic mammalian cells (2, 25). When genomic DNA is released from necrotic cells and disassociated from histones in nucleosomes, it is subjected to demethylation and restriction cleavage (23, 29) to form various demethylated DNA fragments which become effective ligands of TLR9 (30).

However, TLR9 is located in the endoplasmic reticulum and endosomal compartments but not at the cell surface. TLR9 activation by extracellular DNA has to be mediated by a cell-surface receptor. For example, DNA has to gain access through clathrin-dependent endocytosis by binding directly to cell surface receptors such as MARCO and scavenger receptor-1 found in macrophages (31) or RAGE in pDCs as reported in this paper.

A more efficient DNA internalization can occur when more cell surface receptors are recruited through complex formation between DNA and various proteins. For example, the immune complex of
DNA/anti-DNA Abs in SLE provides robust DNA import through FcRII receptor (20). The DNA/HMGB1 complex provides efficient DNA import through TLR2, TLR4, and RAGE receptor (2, 23). In this study, we show that DNA can also complex with complement C3a to act synergistically through a RAGE-dependent pathway to stimulate a strong innate immune response in pDCs and PBMCs.

Genetic deficiency of early complement component C1q, the presence of anti-C1q Ab and accumulation of complement activation product C3a in injured tissues are strongly associated with SLE (9). C1q, recognizing IgM or IgG complex, is critical for activation of classical complement pathway (32). C1q deficiency causes a defect in the clearance of apoptotic cells (33), reduces B cell tolerance to intracellular Ags (34), and predisposes patients to autoimmune disease. Complement C3a is a small 7-kDa protein generated by C3-convertase during complement activation through the classical pathway, MB–lectin pathway, or alternative pathways (35). C3a is one of most potent mast cell chemottractants and induces mast cell degranulation (36). C3a is also a potent inflammatory mediator and stimulates TLR4 and TLR9 responses in pDCs through C3aR-dependent pathway (37) or RAGE-dependent pathway as shown in Figs. 6 and 7.

The role of RAGE in acute and chronic inflammatory diseases has been demonstrated and has been recognized as a therapeutic target (38). RAGE is upregulated in SLE in the acute phase of glomerular injury in lupus nephritis (39). The spliced extracellular domain of RAGE (soluble RAGE) accumulates in the blood of SLE patients, and RAGE expression correlated with SLE disease severity (40). In addition, we demonstrated here that RAGE has high affinity to major pathogenic molecules (e.g., C3a, DNA, HMGB1, AGEs, C3a/DNA complex, and HMGB1/DNA complex) found in SLE. However, RAGE is not the only receptor for these ligands. HMGB1 and HMGB1/DNA complex could gain access to TL9 through its interaction with TLR2 or TLR4 receptors, C3a and C3a/DNA complex through C3aR receptor, and DNA through the MARCO scavenger receptor. In disease, these multireceptor/multiligand pathways may be hard to block. An anti-RAGE Ab for example would not block RAGE-independent pathways that involve ligands that are shared between RAGE and other receptors. Soluble RAGE has an interesting therapeutic hypothesis in that by binding and sequestering ligands, it could block multiple inflammatory pathways and have some therapeutic effect in autoimmune diseases (41). As SLE is a complex autoimmune disease involving various key components of the immune system, targeting multiple pathways might lead to more effective therapeutic approaches.

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References