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Nucleic Acid Sensing by the Immune System: Roles For the Receptor For Advanced Glycation End Products (RAGE) and Intracellular Receptor Proteins: A Dissertation

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NUCLEIC ACID SENSING BY THE IMMUNE SYSTEM: ROLES FOR THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) AND INTRACELLULAR RECEPTOR PROTEINS

A Dissertation Presented By

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14 July, 2011

Desde Quito
hasta el fin de la tierra
yo caminé tu piel,
en ella me interné,
me la aprendí al dedillo.
En ella caí en éxtasis,
de nuevo volvi en mí,
me di las vueltas,
apaciguado a veces;
a veces desbocado,
lúcido hasta más no poder,
sonámbulo, enviciado,
ensimismado, entimismado,
con el tacto borracho,
viviendo en la candela.
Un día
cuando salía el sol
por donde nunca,
con estos ojos
que han de hacerse tierra
yo vi como en tu piel
pastaban las palabras.

-Euler Granda
"Entre la gente y el humo de los carros" de la colección
Relincha el sol, *1997*

From Quito
to the end of the earth
I walked your skin,
I wrapped myself in it,
I learned it down to the last finger.
I fell into the ecstasy of it
came to myself again,
made the rounds,
sometimes appeased;
sometimes out of control,
lucid to the max,
a sleepwalker, an addict,
stuck inside myself, stuck with you,
with a drunken touch,
living in the fire.
One day
as the sun rose
somewhere new,
with these eyes
that will surely turn to dust
I saw that on your skin,
words were grazing.

-Euler Granda, Ecuadorian poet
(Translation is mine. -CMS)

We know very little, and yet it is astonishing that we know so much, and still more astonishing that so little knowledge can give us so much power.

-Bertrand Russell
in his book, ABC of Relativity

in the right light, study becomes insight

-Rage Against the Machine
"Take the Power Back"
from their self-titled album

DEDICATION

For my mother.

Because it was she who repeated,
“I really think you’d like biology,”
when I was convinced that I should go into international relations.
Perhaps we were both right.

ACKNOWLEDGEMENTS

It has been said that it takes a whole village to raise a child. In my case, I feel that it has taken a worldwide community to move me toward this doctorate. The appreciation I show here is a mere gesture, and almost certainly an inadequate one, to recognize the many gifts of time and effort I have received while working toward this dissertation.

I am grateful to my Thesis Research Advisory Committee: Drs. Egil Lien, Kate Fitzgerald, Elizabeth Luna and Hardy Kornfeld, whose guidance has played a large part in the completion of this work. I also extend many thanks to Dr. Ian Rifkin for reviewing my dissertation.

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vielen Dank, tusen takk, go raibh mile maith agat, baie dankie, tack så mycket, grazie mille, merci beaucoup, muito obrigada, köszönöm szépen, dhanyawaad, dank je wel, xie xie, gracias de todo corazón, and thank you very much!

ABSTRACT

As humans, we inhabit an environment shared with many microorganisms, some of which are harmless or beneficial, and others which represent a threat to our health. A complex network of organs, cells and their protein products form our bodies' immune system, tasked with detecting these potentially harmful agents and eliminating them. This same system also serves to detect changes in the healthy balance of normal functions in the body, and for repairing tissue damage caused by injury. Immune recognition of nucleic acids, DNA and RNA, is one way that the body detects invading pathogens and initiates tissue repair. A number of specialized receptor proteins have evolved to distinguish nucleic acids that represent "threats" from those involved in normal physiology. These proteins include members of the Toll-like receptor family and diverse types of cytosolic proteins, all of which reside within the confines of the cell. Few proteins on the cell surface have been clearly characterized to interact with nucleic acids in the extracellular environment. In this dissertation, I present collaborative work that identifies the receptor for advanced glycation end products (RAGE) as a cell surface receptor for nucleic acids and positions it as an important modulator of immune responses. Molecular dimers of RAGE interact with the sugar-phosphate backbones of nucleic acid ligands, allowing this receptor to recognize a variety of DNA and RNA molecules regardless of their nucleotide sequence. Expression of RAGE on cells promotes uptake of DNA and enhances

subsequent responses that are dependent on the nucleic acid sensor Toll-like receptor 9. When mice deficient in RAGE are exposed to DNA in the lung, the predominant site of RAGE expression, they do not mount a typical early inflammatory response, suggesting that RAGE is important in generating immune responses to DNA in mammalian organisms. Further evidence suggests that RAGE interacts preferentially with multimolecular complexes that contain nucleic acids, and that these complexes may induce clustering of receptor dimers into larger multimeric structures. Taken together, the data reported here identify RAGE as an important cell surface receptor protein for nucleic acids, which is capable of modulating the intensity of immune responses to DNA and RNA. Understanding of and intervention in this recognition pathway hold therapeutic promise for diseases characterized by excessive responses to self nucleic acids, such as systemic lupus erythematosus, and for the pathology caused by chronic inflammatory responses to self and foreign nucleic acids.

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List of Abbreviations

AGE	advanced glycation end products
AIM2	absent in melanoma 2
Akt	V-AKT murine thymoma viral oncogene homolog
AP-1	activator protein 1
ATPase	adenosine triphosphatase
BCR	B cell antigen receptor
BS3	bis(sulfosuccinimidyl) suberate
CARD	caspase activation and recruitment domain
CARDIF	CARD adaptor inducing interferon beta
CD11b	cluster of differentiation 11b
CD14	monocyte differentiation antigen CD14
Cdc42	cell division cycle 42
CFP	cyan fluorescent protein
CpG	cytosine-phosphate-guanine dinucleotide
DAI	DNA-dependent activator of interferon regulatory factors
DAMP	damage-associated molecular pattern
DExD/H-box	aspartate-glutamate-any amino acid-aspartate/histidine-box
DHX	aspartate-glutamate-any amino acid-aspartate/histidine-box
DNA	deoxyribonucleic acid
ds	preceding “DNA” or “RNA,” indicates “double stranded”
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FRET	Förster (or fluorescence) resonance energy transfer
gp96	stress-inducible tumor rejection antigen gp96
GRP94	glucose-regulated protein, 94-kD
GTPase	guanosine triphosphatase
HEK293	human embryonic kidney 293 cell line
HIN	hemopoietic IFN-inducible nuclear protein
HLA	human leukocyte antigen
HMGB1	high mobility group box 1
i.e.	*id est* [Latin: that is (to say)]
IFI16	interferon-gamma-inducible protein 16
IFN	interferon
IPS-1	interferon beta promoter stimulator 1
IRAK	interleukin 1 receptor-associated kinase
IRF	interferon regulatory factor
IκB	I kappa B inhibitor of NF-κB
JAK	Janus kinase
LGP2	laboratory of genetics and physiology 2
LPS	lipopolysaccharide

LRR	leucine-rich repeat
LRRfip1	leucine-rich repeat in Flightless-I interacting protein-1
MAL	MyD88 adaptor like
MAMP	microbial-associated molecular pattern
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling
MDA-5	melanoma differentiation-associated gene-5
MEK1	MAPK ERK kinase 1
MHC	major histocompatibility complex
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor kappa B
NLRP3	NOD-like receptor family, pyrin domain containing 3
ODN	oligodeoxynucleotide
ORN	oligoribonucleotide
PAMP	pathogen-associated molecular pattern
PAR	poly-adenosine triphosphate ribose
parylation	poly-adenosine triphosphate ribosylation
pDC	plasmacytoid dendritic cell
PEI	polyethyleneiminie
pH	potential hydrogen
PO	phosphodiester
Poly I:C	polyriboinosinic:polyribocytidilic acid
PS	phosphorothioate
Rab	Ras-associated protein
Rac-1	Ras-related C3 botulinum toxin substrate 1
RAGE	receptor for advanced glycation end products
Ras	Harvey rat sarcoma viral oncogene homolog
Rho	Ras homolog gene family member
RIG-I	retinoic acid-inducible gene I
RIP1	receptor interacting protein 1
RLH	RIG-I-like helicase
RNA	ribonucleic acid
RNA pol III	ribonucleic acid polymerase III
SLE	systemic lupus erythematosis
Src	V-SRC avian sarcoma viral oncogene
ss	preceding “DNA” or “RNA,” indicates “single stranded”
STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes
TBK1	Tank-binding kinase 1
TICAM1	TIR domain-containing adaptor molecule 1
TICAM2	TIR domain-containing adaptor molecule 1
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain-containing adaptor protein

TLR	Toll-like receptor
TRAF3	TNF receptor-associated factor 3
TRAF6	TNF receptor-associated factor 6
TRAM	TIR-containing TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing interferon-beta
UNC93B1	homolog B1 of *C. elegans* UNC93
VISA	virus-induced signaling adaptor
YFP	yellow fluorescent protein

CHAPTER I: Introduction

The mammalian immune system is a complex network of specialized organs and cells that perform surveillance of the body's physical integrity and enact mechanisms to eliminate agents that pose a threat to normal physiological functions. The ability to effectively distinguish "self" from "foreign" and control interactions between the two is what allows us, as humans, to persist for long periods in an environment full of microbes and parasites that wish to benefit from the resources afforded by our bodies. At the same time, we derive great benefit from a large number of commensal microorganisms whose presence, while not strictly "self," does not pose a direct threat to our health and integrity. Given the large number of microorganisms and parasites in our environment, as well as the variety of threats and benefits these organisms pose, the immune system requires sophisticated mechanisms for distinguishing and controlling the interactions of these "foreign" agents with our "self" environment. In recent years, it has become clear that the same immune system that recognizes foreign agents also plays a role in detecting self-derived signals that indicate homeostatic perturbations. Thus, the ability to discern potential danger from normalcy is both complex and essential for survival. Internal errors in the functioning of the immune system can have serious consequences: exaggerated responses to harmless agents, as seen in allergy and hypersensitivities, the mistaken recognition of self as foreign, leading to self-directed attacks known as autoimmunity, and collateral damage to self tissues, termed pathology. Understanding the ways the immune system functions at the molecular level not

only provides insight into the fascinating way that our bodies interact with their environment, but also helps us to devise strategies to correct immune “errors” and create more effective therapies for diseases.

Molecular patterns initiate innate immune responses

As might be expected for a system that must discriminate between a large variety of potential activators, responses of the immune system occur in several interconnected phases. In the broadest of terms, these phases are grouped into those of the early, “innate” immune response and a subsequent “adaptive” response. Both early and late responses are mediated by specialized cells and the effector molecules they produce, but the specificity of these cells and associated proteins differ in important ways. The innate immune response is characterized predominantly by the recognition of conserved “molecular patterns” that are common among certain classes of pathogens (so-called pathogen-associated molecular patterns, PAMPs), microbes in general (microbial-associated molecular patterns, MAMPs) or substances that present themselves during conditions of injury or infection (danger-associated molecular patterns, DAMPs). The chemical nature of these molecular patterns allows them to interact with germline-encoded receptor proteins expressed on the surface of cells or within them, predominantly effector cells of the immune system. Engagement of such innate immune receptor proteins initiates signaling

cascades that result in gene transcription and the production of effector molecules, as well as downstream molecular signals. Many of these signals then converge on additional cell types and receptors with more restricted specificities, which compose the “adaptive” phase of the immune response. While the innate phase rapidly detects signs of infection or damage and begins to control the sources, the adaptive phase completes this process and generates long-lasting immunological memory.

Molecular patterns take a wide variety of chemical forms, including proteins and lipids of bacterial cell walls and membranes, fungal structural proteins, certain carbohydrate conformations, and electrostatically-charged molecules. While many of these “patterns” are highly conserved among certain types of microorganisms, the most highly conserved molecules of all, that is, the basic genetic materials of life, are also a means the immune system uses to sense danger. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) molecules are potent activators of immune responses and tissue repair processes.

Immunostimulatory nucleic acids as molecular patterns

The notion that nucleic acids could stimulate an immune response emerged in the 1960’s, with the realization that viral genetic material induced the production of interferons, molecules that promote changes in cells that “interfere” with viral proliferation.[1] The essential conundrum of distinguishing one’s own nucleic acids

from those of an invading virus was recognized during this early work and is articulated by Rotem and colleagues in a 1963 paper in *Nature*:

> "...if viral nucleic acid is the stimulus to make interferon this poses an awkward problem, since both DNA and RNA viruses are able to induce its production. This suggested a hypothesis -- that the essential stimulus to make interferon might be nucleic acid that was 'foreign' to the cell."[1]

The characteristics that defined RNA and DNA molecules as "foreign" emerged over the next several decades and continue to be refined by recent work. Interferon stimulation was first tied to the presence of long stretches of double-stranded RNA[2], which commonly occur during viral replication but are not native to mammalian cells, where RNA does not persist in a double-stranded state. By the 1970's both natural and synthetic double-stranded RNAs had been identified as stimulators of interferon and the therapeutic potential of synthetic polyriboinosinic:polyribocytidilic acid (poly I:C) against viral infections had been noted[3]. Additional immunostimulatory characteristics of RNA unrelated to double-strandedness emerged more than 30 years after these initial observations, and include the presence of a 3' triphosphate, certain sequence motifs, and particular secondary structures[4]. Recognition of DNA as an inducer of immune responses gained force in the early 1990s, with work that defined DNA-rich mycobacterial extracts as potent instigators of inflammation[5]. These and similar microbial DNA extracts, as well as synthetic oligonucleotides based

on microbial genetic sequences, were shown to have antitumor activity[6] and adjuvant effects, and activate specific subsets of immune cells[7,8]. Many of these studies pointed to specific sequence motifs that appeared to be required for stimulating immune effects, the most elemental of which was an unmethylated cytosine-phosphate-guanine (CpG) dinucleotide contained within palindromic sequences[5,8]. These studies grew into a subfield of research around the therapeutic effects of “antisense” DNA molecules, and the subsequent creation of many synthetic oligonucleotides that have proven useful for activating nucleic acid sensing pathways in experimental and therapeutic contexts.

Toll-like receptors

Discovery

While the RNA and DNA immune activators and their biological effects became more and more defined, an essential link was missing: what molecule or molecules sensed these nucleic acids and instigated cytokine production? In this context, the description by Hemmi and colleagues of a Toll-like receptor protein that mediates responses to bacterial DNA[9] began a period of revelation in the understanding of nucleic acid sensing. This DNA-binding Toll-like receptor, TLR9, recognizes double-stranded (ds) and single-stranded (ss) DNA by means of interaction with unmethylated cytosine-phosphate-guanine (CpG) dinucleotide motifs and surrounding nucleotide bases. Optimal CpG motif sequences

generally consist of a cytosine followed directly in the 3' direction by a guanine and surrounded by particular combinations of purine and pyrimidine bases. Such motifs are common in bacterial and viral genomes, but are infrequent and methylated in the human genome. A wide range of synthetic oligonucleotides incorporating CpG motifs and stabilizing structural elements have been developed that are able to selectively induce and block TLR9 signaling[10-13].

At the time that TLR9 was described, the Toll-like receptor field was blossoming. The description in 1997 of the first human homolog of the Drosophila receptor Toll, TLR4, and the realization that it mediated mammalian responses to the potent bacterial PAMP, lipopolysaccharide (LPS)[14-16], revolutionized the study of innate immunity. Seemingly in a heartbeat, genes for five structurally-related TLRs were defined and named TLR1 through TLR5[17], and TLR6 was described soon after[18]. All of these new TLRs were assumed to play roles in immune pattern recognition[19], but identification of their specific ligands lagged behind their molecular cloning. On the heels of TLR4, TLR2 was found to recognize bacterial membrane lipids distinct from LPS[20-24] and TLRs 1 and 6 were subsequently shown to heterodimerize with TLR2 in response to subsets of these PAMPs[25,26]. A flurry of research surrounding TLRs 2 and 4 had firmly established a key role for this receptor family in sensing of bacterial components. The description of TLR9 as a receptor for bacterial DNA thus not only contributed another member to the panel of bacteria-detecting receptors, but also indicated that the first clear DNA-sensing receptor protein was a TLR. Soon

thereafter, dsRNA was defined as a ligand for TLR3[27], thus consolidating a role for TLRs in nucleic acid sensing and serving as a link between immunostimulatory nucleic acids and cytokine production.

Protein structure

Toll-like receptors are a family of structurally-related transmembrane proteins, possessing extracellular (or simply, “ecto-”) domains that contain a series of repeating leucine-rich sequences (“leucine-rich repeats” or LRRs), a single hydrophobic transmembrane region, and a cytosolic domain with homology to members of the interleukin-1 receptor family, termed the Toll IL-1 receptor (TIR) domain. Variations in the number and length of LRRs have led to classification of the TLRs into structural subfamilies[28], while homology of the TIR domains allows for homotypic association with TIR domains of downstream adaptor molecules that facilitate signal transduction[29]. A total of 13 TLRs have been defined in humans (TLR1-10) and mice (TLR1-9, 11-13), while other vertebrate and invertebrate animals possess diverse and less-studied repertoires[30-32].

Endosomal TLRs

TLR9 was cloned and described together with two other novel Toll-like receptors, dubbed TLR7 and 8[33,34] and these three receptors were shown to share a longer ectodomain structure, distinguishing them as a structurally-distinct subfamily from the six previously described TLRs. TLRs 7-9 were subsequently recognized to

differ in another key way: they were expressed on intracellular membranes, rather than at the cell surface. TLRs 1, 2, 4, 5, 6 and 10 are all expressed on the plasma membrane, while TLRs 7, 8, 9, along with TLR3[*1], are expressed in the endoplasmic reticulum of resting cells. Upon appearance of ligands, these intracellular TLRs relocate to endosomal compartments, and are thus sometimes referred to collectively as endosomal TLRs. Interestingly, as ligands for the remaining endosomal TLRs emerged, it became clear that these receptors had something else in common: they all recognize nucleic acids. TLR3 was found to sense viral dsRNA[27] and this recognition extended to synthetic dsRNA molecules like poly I:C. The first defined ligands for TLRs 7 and 8 were synthetic ribonucleoside analog drugs[35], but the ability of these receptors to recognize specific types of viral or synthetic ssRNA eventually became clear[36].

Endosomal localization in TLR function

The fact that nucleic acid sensing TLRs are all grouped into the same intracellular membrane-bound compartments would seem to suggest that this localization is vital for effective sensing of foreign nucleic acids. As the environment in which all TLR:nucleic acid functional interactions appear to occur is within endosomes, it helps to consider some generalities of endosome biology[37] when contemplating nucleic acid sensing in this environment. The main function of the endosomal network is to transport materials -- macromolecules,

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[1] TLRs 11[257] and 13[258] are also expressed on intracellular membranes

whole or fragmented microorganisms, crystalline materials, etc.-- from the extracellular environment into the cell. Formation of an endosome generally requires a signal from proteins on the cell surface that recognize potential endosome “cargo,” processes known as receptor-mediated endocytosis and phagocytosis. Passive uptake of some receptor proteins and soluble materials in extracellular fluid also occur constantly as a result of pinocytosis. Endocytosis initiates when a region of plasma membrane buds inward, enclosing membrane-bound proteins and cargo components in a vesicle, which soon fuses with a larger endosome near the cell surface. As a result, the endosome lumen is, essentially, a continuation of the “extracellular” environment, though no longer in direct contact with it. Hence, the lumen-exposed “ectodomains” of TLRs 3, 7-9 are functionally similar to the “extracellular” domains of TLRs 1, 2, 4-6. Endosomes that fuse directly with vesicles from the plasma membrane are called “early” endosomes and are characterized by an acidic pH of around 6. This pH is maintained by hydrogen-transporting ATPases and often facilitates release of cargo from receptor proteins. Some of these proteins will be transferred to vesicles that fuse with distinct, “recycling” endosomes for return to the cell surface. Other receptors, and nearly all endosome cargo, remain in the acidified compartment, which through a number of fusion events, matures into a “late” endosome. Endosomal maturation is a dynamic process characterized by vesicle movement along the cytoskeleton and iterative changes in membrane protein composition. Certain proteins, most notably those of the Rab GTPase

family, are thought to form “labels” that promote recognition of different types of endosomes by other vesicular bodies[38]. These endosomal “markers” can be exploited to identify specific endosomal compartments under experimental conditions. Fusion of late endosomes with vesicles containing acid-dependent proteases and lipases forms a degradative compartment known as the lysosome or endolysosome, in which microbes and macromolecules are broken down.

The acidification of the endosomal compartment has been shown to be essential for activation of TLR9 by DNA ligands[39,40], and similar requirements appear to exist for activation of TLR7 and 8 by their ligands[41]. Several hypotheses have been put forth to explain why nucleic acids must meet TLR9 in an acidified endosome for activation (refer to [42] and [43]):

1. Low pH and activation of acid-dependent degradative enzymes modify ligands in a manner necessary for their recognition by the receptor. While this may be true for DNA molecules themselves, it would seem to be of greater importance for extracting nucleic acids from viral particles, bacterial cells or parasites. Similarly, these chemical changes might help to physically separate DNA from delivery vectors such as malarial hemozoin[44], artificial transfection agents, or co-receptor proteins.
2. Acid-dependent proteases directly modify TLR9 to enable receptor engagement by ligands. This idea, initially put forth by Ploegh and colleagues[45], has been a controversial one. It now appears that, although

TLR9 cleavage is not required for ligand binding, it is important for downstream signaling of endogenous TLR9[46]. Some indirect evidence indicates that a similar cleavage mechanism could also affect TLR7 activation[47].

3. The restricted dimensions and specific chemistry of the endosome serve to concentrate TLR 9 ligands, thus surpassing an activation threshold.
4. Its ability to enter an endosomal compartment serves as an indicator that a given nucleic acid molecule requires the attention of the immune system. Since a healthy cell's own genomic DNA is confined to the nucleus and its RNA functions in either the nucleus or the cytosol, sequestering nucleic acid-sensing TLRs in endosomes restricts their access solely to nucleic acids originating from extracellular sources.

Support for this final hypothesis was convincingly developed in work by Barton and colleagues[42]. In an elegant series of experiments, they showed that swapping the transmembrane and intracellular domains of TLR9 with TLR4 directed the chimeric TLR9 protein to the plasma membrane and enabled it to recognize self-derived DNA in the extracellular milieu. This strongly supported the idea that sequestration of TLRs in endosomal compartments was the key determinant in preventing their activation by self nucleic acids. At the same time, the surface-expressed TLR9 was no longer capable of recognizing genomic DNA from the virus HSV-2, presumably because this DNA was protected by the viral capsid when in the extracellular environment. Thus, endosomal localization of

TLR9 not only prevented its interaction with extracellular self DNA but also promoted its access to infectious viral DNA. This latter point lends support for the role of degradation in the lysosome (hypothesis 1, above). Moreover, the fact that surface-exposed TLR9 could effectively respond to low concentrations of DNA in the extracellular environment suggests that the ability to concentrate DNA (hypothesis 3) is not an essential role of the endosome for TLR9 function.

Biology of endosomal TLRs: insights from autoimmune disease

Although sequestration of TLRs in endosomes appears to largely prevent self-recognition, involvement of intracellular TLRs has been described in certain autoimmune pathologies. Antibodies that can bind chromatin, RNA and associated nucleoproteins form “immune complexes” that are characteristic of the autoimmune disease systemic lupus erythematosus (SLE). Such immune complexes can be endocytosed via interaction with cell surface receptors that recognize the Fc region of the antibodies, thus facilitating the uptake of complexed nucleic acids into endosomes and activation of innate antigen presenting cells[48-50]. B lymphocytes express a specialized membrane-bound antibody known as the B-cell antigen receptor (BCR) and are able to recognize and endocytose immune complexes via BCR engagement. Synergy between BCR-derived and TLR7/9-derived signals are required for B-cell activation by nucleic acids[51,52]. Immune complex diseases involve multiple types of immune cells and engage several signaling pathways. Interestingly, there is evidence

that interplay between nucleic acid sensing TLRs modulates SLE-like pathology in mice, with TLR7 generally exacerbating disease and TLR9 partially mitigating TLR7-mediated effects[53]. Thus, understanding the interconnections between nucleic acid receptors and their modulation is a key step in preventing and treating certain types of autoimmunity.

Signaling

Signaling pathways initiated by endosomal nucleic acid-sensing TLRs are common to all studied TLRs and are divided into two branches: the MyD88-dependent and MyD88-independent pathways (**Figure 1.1**) The MyD88-dependent pathway is used by all studied TLRs with the exception of TLR3[54,55]. The pathway takes its name from the essential role of the TIR-containing adaptor protein myeloid differentiation primary response gene 88 (MyD88), which associates via its TIR domain with the TIR domains of TLRs. In the case of TLRs 2 and 4, this interaction is bridged by another TIR-containing adaptor, MyD88 adaptor like (MAL)[56-58 *2] , while TLRs 5, 7-9 appear to associate with MyD88 directly[59-61]. Association of MyD88 with TLRs is followed by the recruitment of IL-1 receptor associated kinases (IRAK family members) to form a receptor complex known as the “Myddosome”[61,62]. IRAKs are then able to recruit the ubiquitin ligase TNF-associated factor 6 (TRAF6)[59], which can activate the

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**2 MAL is also called TIR domain-containing adaptor protein (TIRAP)*

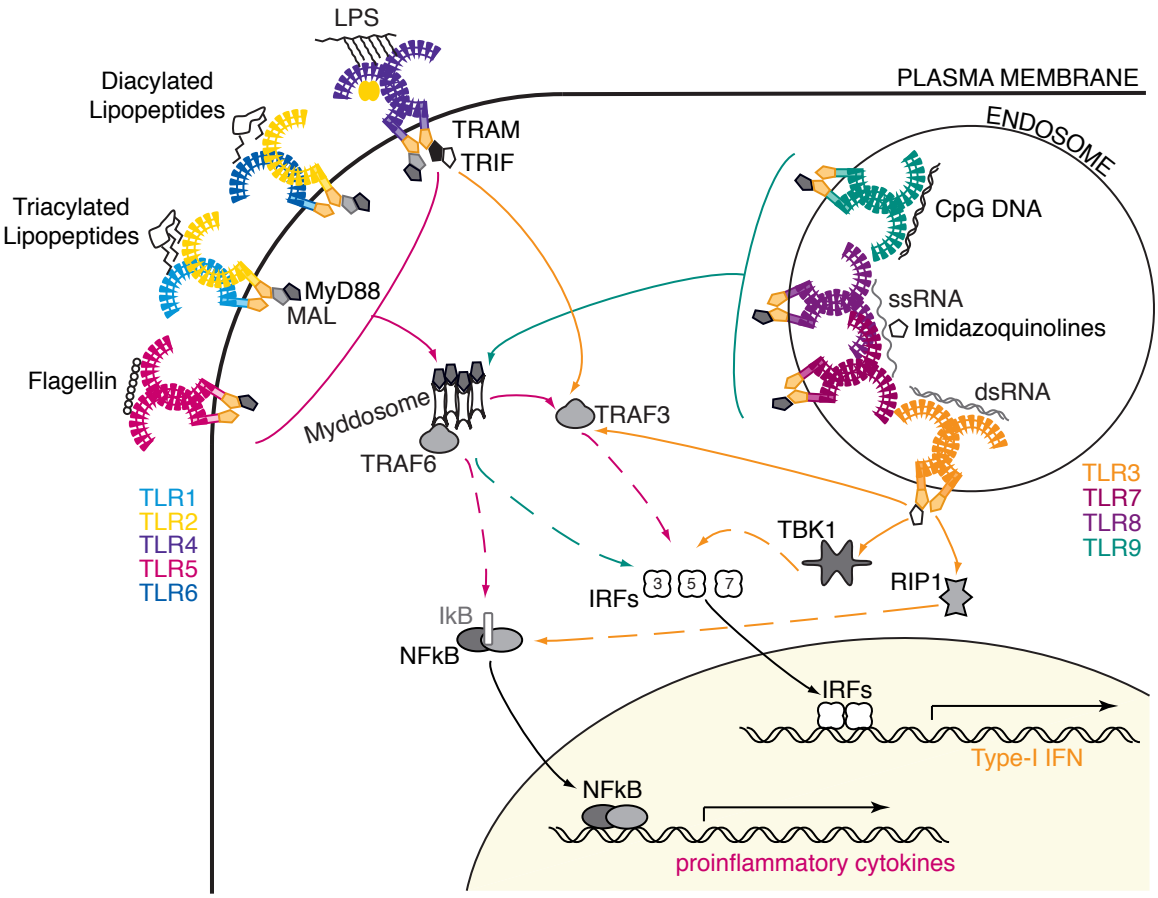

Figure 1.1 Toll-like receptors, their ligands, and simplified signaling pathways.
TLRs 1-2, 4-6 are expressed on the cell surface, while TLRs 3, 7-9 are expressed in intracellular compartments (TLRs 10-13 are not shown). Upon ligand binding, TLRs initiate signaling cascades involving the adaptor protein MyD88 (magenta and green lines) or the adaptor protein TRIF (orange lines). Dotted lines represent simplified pathways with known intermediates. “Myddosome” refers to a multimolecular complex of MyD88 with varying combinations of IRAK1, IRAK2 and IRAK4. For explanation of abbreviations please refer to the text and the List of Abbreviations.

transcription factor nuclear factor κB (NF-κB), mitogen-activated protein (MAP) kinases[63] and interferon regulatory factor 5 (IRF5)[64,65], leading to transcriptional upregulation of proinflammatory cytokines. IRAK 1 activation can also lead to production of type-I interferon (IFN) via activation of TRAF6[66] and the related protein TRAF3[67], which activate other members of the IRF family of transcription factors.

The MyD88-independent signaling pathway used by TLR3 involves interaction of the TLR TIR domain with that of TIR domain-containing adaptor inducing interferon-beta (TRIF)[68,69 *3]. This leads to activation of TRAF3 and the Tank binding kinase 1 (TBK1) complex, which phosphorylates IRFs 3 and 7, resulting in upregulation of type-I IFN[67]. TRIF can also activate the kinase receptor interacting protein 1 (RIP1), which leads to activation of NF-κB and transcriptional activation of proinflammatory cytokines[70]. TLR4 also activates these TRIF-dependent signaling pathways, and associates with TRIF with the help of TIR-containing TRIF-related adaptor molecule (TRAM)[71,72 *4]. Hence, TLR4 is the only TLR which signals through both MyD88 and TRIF, and requires the adaptors MAL and TRAM in order to do so. Because all known human and murine TLRs signal via MyD88 and/or TRIF, animals doubly deficient in these two adaptor proteins[55] are considered null for all TLR-based signals and these

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**3 TRIF is also called TIR domain-containing adaptor molecule 1 (TICAM1)*

**4 TRAM is also known as TIR domain-containing adaptor molecule 2 (TICAM2)*

animals are frequently used to distinguish TLR-mediated versus TLR-independent responses to nucleic acids.

Cytosolic nucleic acid sensors

Targeted delivery of nucleic acids to endosomes by immune complexes, viruses or cationic transfection agents is an important safeguard in restricting the activation of nucleic acid-sensing TLRs. However, many RNA and DNA viruses as well as certain bacterial pathogens penetrate directly into the cytosol of the cell. It was soon recognized that the interferon-stimulating properties of nucleic acids described in the 1960's could not be entirely explained by the action of Toll-like receptors alone. The identification of a cytosolic helicase protein that could activate interferon in response to RNA[73] started off a second "boom" in nucleic acid sensing research, this time in discovery of soluble receptor proteins.

RNA sensors

This first recognized cytosolic sensor, retinoic acid inducible gene I (RIG-I) and the structurally homologous melanoma differentiation-associated gene-5 (MDA-5) are proteins containing an RNA-binding aspartate-glutamate-any amino acid-aspartate/histidine-box (DExD/H-box, or simply DHX) helicase domain and two caspase activation and recruitment domains (CARDs)[73,74], which were later found to interact with the mitochondrially-localized adaptor protein, mitochondrial

antiviral signaling (MAVS)[75-78] *5 (**Figure 1.2**). Both the helicase domain[73] and the ability to interact with MAVS[75] have been shown to be essential for type-I interferon activation. A third member of this RIG-I like helicase (RLH) family, laboratory of genetics and physiology 2 (LGP2), can bind RNA by virtue of a helicase domain, but lacks CARDs to initiate downstream signaling[74]. Thus, is not considered a true RNA sensor, but it may serve to facilitate recognition of RNA by other RLH family members[79]. RLH helicase domains can bind both ds and ssRNA and additional shared and distinguishing features of RLH ligands have been an active area of research. While a number of distinct ligands have been described, current evidence suggests that the optimal RIG-I ligand is blunt-end 5‘ triphosphate-containing dsRNA (refer to [80] and references therein). The ligands for MDA-5 and LGP2 remain largely undefined.

DNA sensors

Similar to RLH, cytosolic receptors mediate responses to DNA in a manner independent of endosomal TLRs. Several cytosolic DNA sensors have been described, and they can be grouped by the signaling pathways they activate (**Figure 1.2**):

**5 MAVS has also been named virus-induced signaling adaptor (VISA), interferon-beta promoter stimulator 1 (IPS-1) and CARD adaptor inducing interferon-beta (CARDIF).*

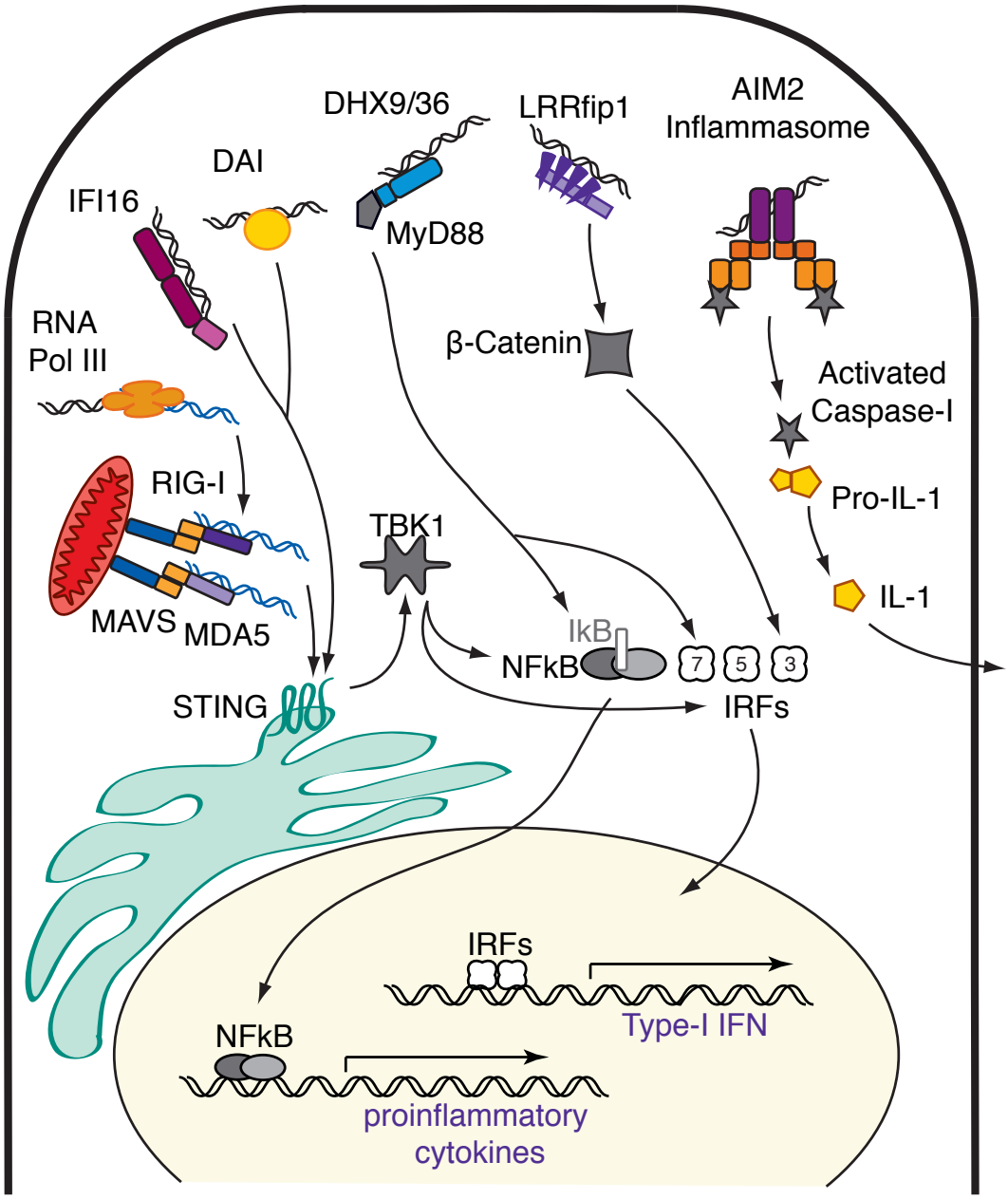

Figure 1.2 Cytosolic nucleic acid receptors and simplified signaling pathways.
Soluble receptor proteins recognize RNA (blue helices) or DNA (black helices) in the cytosol and initiate signaling cascades that converge upon activation of NF-κB and IRF transcription factors to promote the production of proinflammatory cytokines and type-I interferon. For explanation of abbreviations please refer to the text and the List of Abbreviations.

1. Receptors such as DAI[81] [*6] and IFI16[82] induce expression of proinflammatory cytokines and type-I interferon upon DNA ligation through signaling pathways involving STING and either NF-κB (for proinflammatory cytokines) or IRFs (for interferon).
2. Helicases such as DHX9 and DHX36 interact directly with DNA and MyD88 (see discussion of TLR signaling, above) to activate NF-κB and IRF7[83]. Thus, these cytosolic helicases appear to activate production of both proinflammatory cytokines and type-I IFN through MyD88-dependent but TLR-independent pathways.
3. RNA polymerase III (RNA pol III) reverse-transcribes certain types of dsDNA, such as AT-rich DNA, to an RNA ligand that activates RIG-I to induce expression of type-I IFN[84,85].
4. Interferon induction in response to viral and bacterial DNA is induced by leucine-rich repeat in Flightless I interacting protein-1 (LRRfip1) via activation of beta-catenin, which enhances IRF3 activation via a coactivator pathway involving CBP/p300[86] [*7].
5. Upon binding of DNA, the pyrin and HIN domain-containing receptor absent in melanoma 2 (AIM2), forms an inflammasome complex capable of activating the pro-inflammatory cytokine interleukin (IL)-1β[87].

**6 The role of DAI as a DNA sensor is somewhat controversial. Although it was the first identified cytosolic DNA receptor, deficiencies in DNA sensing in DAI-knockout animals have not been clear.*

[7] It is also worth noting that LRRfip1 may sense dsRNA (refer to [259]).

DNA ligands for these receptors are primarily double-stranded and rich in adenine and thymine bases. Certain viral genomes and synthetic molecules have also been used to stimulate particular receptors. However, the essential chemical characteristics of these ligands are yet to be clearly defined.

Accessory molecules for intracellular nucleic acid sensors

A key task in understanding immunity is defining which proteins are "essential" for a given recognition or signaling process and which ones exert enhancing or limiting effects. The TLRs and most [*8] cytosolic receptors mentioned heretofore are all "essential" in the response to particular ligands, though the consequences of their activation are sometimes redundant. Reductionist approaches have supplied us with a good understanding of the mode of action of these essential receptors. However, as the naturalist John Muir has noted, "when we try to pick out anything by itself, we find it hitched to everything else in the universe"[88]. Nucleic acid receptors are no exception to this axiom and several macromolecules have emerged as accessory factors that contribute to proper receptor function.

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[8] *To date, a complete functional deficiency in the absence of DAI, LRRfip1 or the DHX helicases has not been clearly demonstrated.*

Though negative regulation is certainly important for controlling inflammatory reactions, such downregulation of nucleic acid sensors happens primarily at the level of receptor gene transcription or regulation of downstream signaling intermediates. Here, I will focus on factors that promote receptor activation via direct (or potentially direct) molecular interactions.

Proteins that promote proper receptor localization

All TLRs are transmembrane proteins. As such, they are synthesized on the membrane of the endoplasmic reticulum (ER) and then travel to other membrane-bound compartments or the plasma membrane via the cell's vesicular transport network. The proper folding of TLRs as they are synthesized in the ER has been shown to depend on the chaperone protein gp96 [*9]. Loss of gp96 function ablates the ability of cells to respond to TLR ligands[89], suggesting that creation of a TLR "stock" in the ER requires the help of this chaperone. Similarly, at least two ER-resident proteins appear to be important for nucleic acid sensing TLRs to translocate from ER stores to endosomes. Absence of the ER lumen protein associated with TLR4, A (PRAT4A) [*10] appears to impede the ability of TLR9 to effectively translocate to the endosome[90]. Cells deficient in PRAT4A also showed decreased ability to respond to a TLR7 ligand, but not to a TLR3 ligand[90], suggesting that this protein may be key for transport of endosomal TLRs

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[9] gp96 is also known as glucose regulated protein-94 kD (GRP94)

*[*10] PRAT4A is also called trinucleotide repeat-containing gene 5 (TNRC5)*

that ultimately interact with MyD88, but is not essential for endosomal TLR trafficking that activates the TRIF-mediated pathway. A membrane-embedded ER protein, UNC93B1, however, seems to be a master regulator of all ER-to-endosome translocation. A single point mutation in this protein keeps it from interacting with TLRs 3, 7 and 9[91], which confers a “triple deficient” (“3d”) phenotype in mice[92]. Subsequent work has confirmed that UNC93B1 travels with TLRs from the ER to endosomes[93] and that this protein may also exert other regulatory effects on endosomal TLR function[94]. Beyond the fundamental ER-to-endosome transport event, at least one protein may be necessary for mediating the translocation of TLR9 from a strictly endosomal compartment to a more mature endolysosomal compartment. Adaptor protein 3 (AP3) is required for the production of type-I IFN but not for activation of NF-κB downstream of TLR9 in plasmacytoid dendritic cells (pDC), suggesting that distinct signals emanate from TLR9 depending on the maturity of the endosomal compartment in which it resides[95]. However, further work has clarified that the peptide transport protein Slc15a4 may work upstream of AP-3 to maintain essential characteristics of acidfied compartments in pDC[96], and thus these two proteins may play distinct or overlapping roles in regulation of type-I IFN induction by endosomal TLRs.

Regulators of protein production and trafficking have not been clearly identified for cytosolic nucleic acid receptors and the roles of such accessory molecules are likely to be forthcoming.

Cofactors that promote ligand-receptor interaction

TLRs expressed on the cell surface have a number of well-defined co-receptor molecules which facilitate their interactions with ligands[97], and many of these co-receptors are essential for ligand recognition. In contrast, no required co-receptors have been recognized for endosomal TLRs, though several accessory molecules appear to enhance ligand:receptor interactions and/or signaling.

CD14 is a required co-receptor for LPS and lipoprotein recognition by surface TLRs 2 and 4[98,99]. It exists in both membrane-anchored and soluble forms and both of these forms seem to be able to exert co-receptor function for surface TLRs. Recent work has shown that CD14, while not strictly required, also serves as an important cofactor for endosomal TLRs[100,101], where it appears to play roles in ligand uptake and TLR recognition, as well as in enhancing downstream signaling[101].

CD14 itself is not a signaling receptor; however, bona fide plasma membrane signaling receptors have also been proposed to be important for the uptake of nucleic acid ligands into endosomal compartments. These include several proteins common on the surface of phagocytes, such as integrins and scavenger receptors[102-104]. It thus appears clear that a variety of cell-surface proteins can bind nucleic acids and may promote their access to endosomal TLRs. However, a lack of rigorous study of these nucleic acid:receptor interactions at the biochemical level, as well as functional redundancy for nucleic acid uptake that keeps any single receptor from being considered “essential,” has

impeded these receptors from being considered an important component of nucleic acid sensing. Thus, key roles of an “uptake receptor” for nucleic acids still remain to be identified.

In addition to membrane associated co-receptor molecules, regulatable soluble factors that bind nucleic acids have also been suggested to promote immune activation. A cathelicidin antimicrobial peptide with an alpha-helical structure, LL37, has been shown to bind DNA and induce formation of multimolecular complexes[105]. This complexation seems to promote DNA endocytosis, thus enhancing DNA recognition by TLR9[105]. A similar complexation effect is seen with the chromatin binding protein high mobility group box 1 (HMGB1)[106 *11]. When released from cells under conditions of necrosis or cell stress, HMGB1-DNA complexes appear to interact with cell surface receptors including RAGE (this receptor is the focus of the next section). The DNA-TLR9 co-receptor effects of both HMGB1 and LL37 have been shown primarily in plasmacytoid dendritic cells (pDC)[105,106], the key cell type producing type-I IFN in both mice and humans. Interestingly, both LL37 and HMGB1 have been implicated in the enhancement of autoimmune syndromes (ref [107]), suggesting that they are capable of making self DNA more immunogenic. Natural cofactors that promote interaction of extracellular nucleic acids with cytosolic RNA and DNA receptors have not been described. However, the fact that complexation of nucleic acids with synthetic transfection agents is an effective mechanism for

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[11] *HMGB1 is also called amphoterin.*

delivering immunogenic nucleic acids to cytosolic receptors suggests that natural factors with similar capabilities could play important roles in promoting activation of these receptors. Conversely, avoiding uptake of extracellular nucleic acids into the cytosol is likely an important mechanism for ensuring activation of these signaling pathways exclusively by viruses and intracellular pathogens. This idea is reinforced by studies showing that a deficiency of nucleases that degrade endogenous excesses of nucleic acids leads to the strong immunopathology seen in SLE[108], Aicardi-Goutières syndrome and chilblain lupus[109].

All together, our current understanding of the modulators of nucleic acid sensing suggest that the balance between limiting access of nucleic acids to intracellular sensors and facilitating this access is central to recognition of DNA and RNA by the immune system.

RAGE

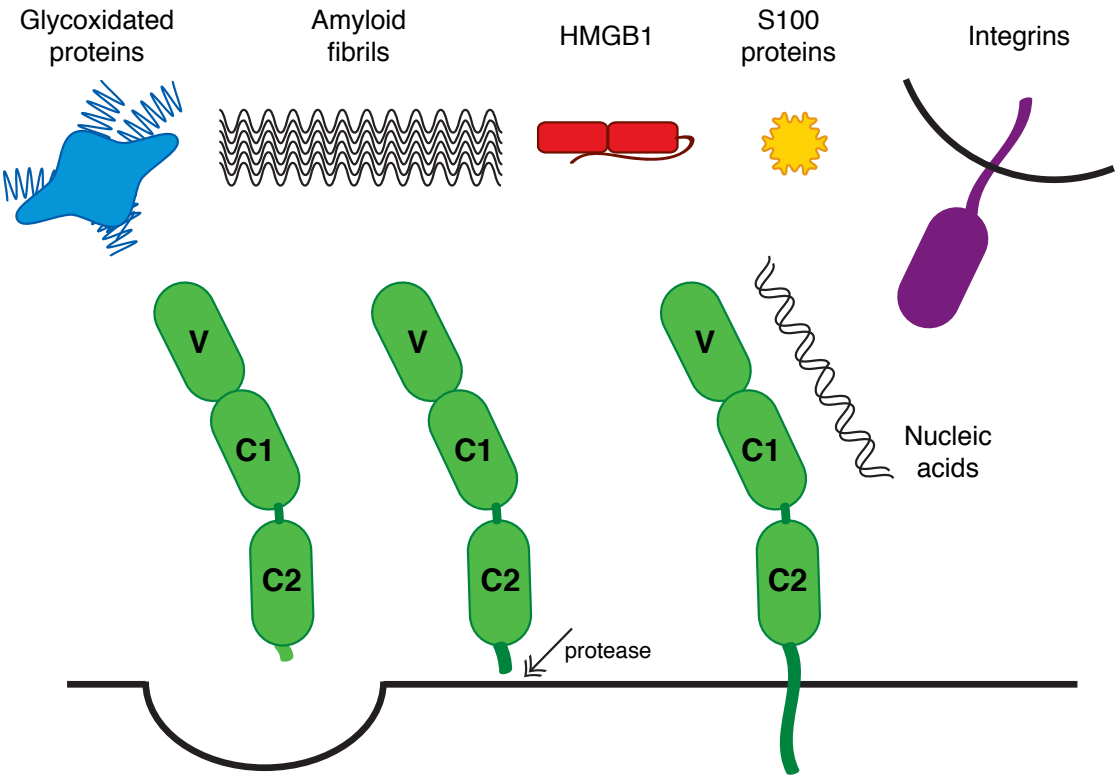
The term “pattern recognition receptor,” while often bringing to mind the well-studied TLRs and RLRs, encompasses many receptor proteins that recognize ligands by means of conserved molecular patterns. One lesser known PRR is the receptor for advanced glycation end-products (RAGE). This plasma membrane protein was initially thought to serve as a scavenger receptor that aided in the clearance of non-enzymatically glycated proteins (advanced glycation end products, AGE) from blood serum (refer to [110]). While its role in

responding to AGE is an important one, this protein does not function specifically to clear these products from the circulation. Instead, RAGE appears to aid in the uptake and initiation of inflammation in response to the presence of AGE and a variety of other endogenously-derived DAMPs.

Gene and protein

The location of the gene encoding RAGE, *ager*, in the human genome is suggestive of a function in immunity. *ager* is located in the human leukocyte antigen (HLA) locus on chromosome 6, near the gene encoding major histocompatibility complex three (MHC III). Structurally, RAGE belongs to the immunoglobulin receptor superfamily and has a conformation similar to that of an antibody heavy chain. RAGE has three extracellular domains named according to their homology with immunoglobulin variable and constant regions, called V, C1 and C2 (**Figure 1.3**). The RAGE "variable" domain does not vary in sequence, however, and recent work has suggested that this domain is more similar to those of adhesion molecules (also immunoglobulin superfamily members)[111]. Following a hydrophobic transmembrane domain is a short cytosolic tail consisting of just 41 amino acids and no clear signaling domains. This full-length membrane-bound RAGE is just one of several naturally-occurring isoforms, which seem to number approximately 20 in humans[112] and in mice[113], though not all isoforms are shared between the two species[113]. Defining a clear

Figure 1.3 RAGE isoforms and ligands. Established RAGE ligands are shown in cartoon form with the secreted, membrane-cleaved and membrane-bound isoforms of RAGE protein (green). V- and C-type immunoglobulin-like RAGE domains are indicated on the cartoon and on the corresponding amino-acid sequence for membrane-bound RAGE. Exon arrangement in cDNA of soluble and membrane-bound isoforms (based on [112]) shows an area of alternative splicing resulting in an altered version of exon 9 (gray box) and an alternative C-terminal amino acid sequence in the secreted protein.



Amino acid sequence:

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                    10                  20                  30                  40                  50                  60
MAAGTAVGAW VLVLSLWGAV VGAQNITARI GEPLVLKCKG APKKPPQRLE WKLNTGRTEA
                    70                  80                  90                 100                 110                 120
WKVLSPQGGG PWDSVARVLP NGSLFLPAVG IQDEGIFRCQ AMNRNGKETK SNYRVRVYQI
                   130                 140                 150                 160                 170                 180
PGKPEIVDSA SELTAGVPNK VGTCVSEGSY PAGTLSWHLD GKPLVPNEKG VSVKEQTRRH
                   190                 200                 210                 220                 230                 240
PETGLFTLQS ELMVTPARGG DPRPTFSCSF SPGLPRHRAL RTAPIQPRVW EPVPLEEVQL
                   250                 260                 270                 280                 290                 300
VVEPEGGAVA PGGTVTLTCE VPAQPSPQIH WMKDGVPLPL PPSPVLILPE IGPQDQGTYS
                   310                 320                 330                 340                 350                 360
CVATHSSHGP QESRAVSISI IEPGEEGPTA GSVGGSGLGT LALALGILGG LGTAALLIGV
                   370                 380                 390                 400
ILWQRRQRRG EERKAPENQE EEEERAELNQ SEEPEAGESS TGGP
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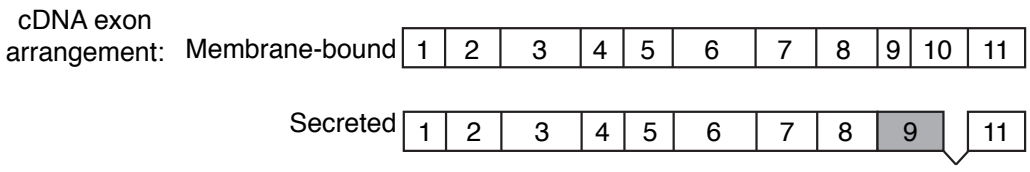

Figure 1.3 RAGE isoforms and ligands.

count of isoforms has been complicated by evidence that post-transcriptional control of RAGE varies across tissues and cell types and that several potential mRNA variants are candidates for the nonsense-mediated decay pathway, and thus may never translate into functional proteins[112,113]. That said, the two predominant forms of RAGE protein detectable *in vivo* are the full-length membrane-bound receptor described above, and soluble forms consisting of only the V-C1-C2 extracellular domains. These soluble isoforms originate from two post-transcriptional processes: alternative mRNA splicing[114-116] and cleavage of the membrane-bound protein by extracellular proteases[117-119]. Because soluble RAGE (sRAGE) has the same ligand binding regions as the membrane-bound form, it is thought to function as an endogenous decoy receptor and this function has been exploited in experimental contexts to block effects mediated by the transmembrane receptor.

RAGE ligands

RAGE:ligand interactions are thought to occur primarily with the outermost V receptor domain (this topic is addressed in detail in Chapter II), and six families of ligands have been clearly established. As previously mentioned, non-enzymatically glycoxidated adducts on proteins were the first recognized RAGE ligands and were the basis for the receptor's name[120]. Many proteins can become covalently decorated with AGE moieties such as (carboxymethyl)

lysine[121] and pronyl-glycine[122] when in the presence of aldose reducing sugars (*i.e.*, glucose) in the bloodstream. Though not technically glycation products, oxidation products such as oxidized low-density lipoprotein (oxLDL)[123] and advanced oxidation protein products (AOPP)[124] form under similar conditions as AGES and are also RAGE ligands. Thus, AGEs are a heterogeneous class of endogenously-formed ligands.

The importance of RAGE as a true pattern-recognition receptor became more clearly defined as additional ligands were identified. Fibrillar forms of amyloid-β were shown to interact with RAGE, which is expressed on neurons and microglial cells in the brain[125]. Both AGEs and amyloid plaques are materials that can accumulate endogenously, and that require removal to maintain normal homeostasis. Hence, RAGE seemed to play a role in detecting accumulations of toxic metabolic products. This role broadened further with the identification of two other ligand classes: proteins of the high mobility group box (most notably HMGB1)[110] and the S100 calcium binding proteins (specifically, S100A12 and S100b)[126]. S100s and HMGB1 are not accumulated metabolites, but rather endogenous molecules released from activated cells during inflammatory processes. RAGE now appeared to be a key sensor of several homeostatic perturbations. A specific role in inflammation was further strengthened by the realization that RAGE could serve as a counterreceptor for β2 integrins, such as CD11b, through homotypic interactions, thus participating in the process of leukocyte recruitment[127]. The most recently defined RAGE ligand,

extracellular DNA[128] (also refer to [129] and data presented in chapter II), bolsters the case that RAGE is integral to inflammation and tissue maintenance, as sensing of extracellular DNA is a key mechanism for inducing immunity and tissue repair processes. The interaction of RAGE with many of these ligands has been studied at the biochemical level and the available evidence indicates that RAGE interacts with these ligands directly. However, potential roles of co-receptors (refer to [130] and [131]) have not been strictly interrogated and additional complexity may emerge to enrich our understanding of RAGE:ligand interactions.

Signaling

The functional consequences of ligand interactions with RAGE have been a topic of intense interest. Early in its identification as the AGE receptor, RAGE was found to induce cellular changes consistent with a role as a signaling receptor[132,133]. Subsequent work has begun to elucidate a complex network of signals that can be initiated by RAGE ligands and the majority of these pathways seem to converge, ultimately, on the activation of the transcription factor NF-κB (**Figure 1.4**). As opposed to the relatively transient nature of NF-κB activation by other PRRs, RAGE-mediated signals seem to lead to a prolonged upregulation of NF-κB over periods of days or weeks, due in part to enhanced *de novo* synthesis of the transcription factor itself[134], as well as increased receptor expression due to NF-κB activation of the RAGE promoter[135]. The receptor-proximal factors that connect RAGE to activation of NF-κB include elements of several distinct

Figure 1.4 Signaling pathways activated by RAGE. Upon ligand binding, RAGE initiates distinct and intersecting signaling cascades to activate NF-κB and other transcription factors, as well as cytoskeletal remodeling. Initiation of signaling may involve caveolin-1 (Cav-1) and diaphanous-1 (Dia1) as adaptors, or direct interaction of RAGE with downstream kinases and GTPases. For complete explanation of abbreviations, please refer to the text or the List of Abbreviations.

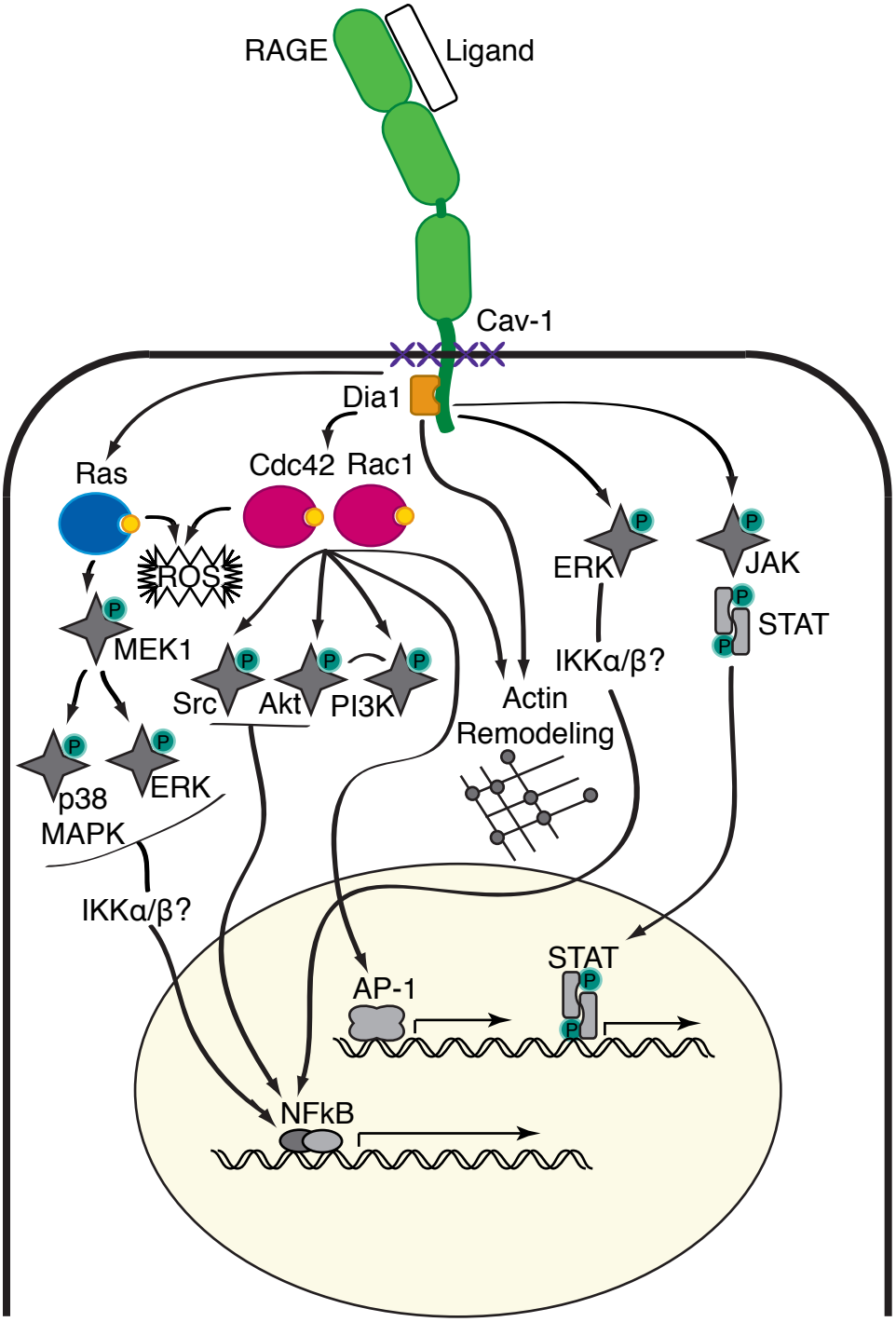

Figure 1.4 Signaling pathways activated by RAGE.

signaling pathways and the pathway activated may depend to some extent on the type of cell expressing RAGE, as well as the ligand activating the receptor. In several cell types, Rho-family GTPase proteins Rac-1 and Cdc42 become activated by RAGE[136-138]. Once converted to their GTP-bound active state, these proteins have been shown to activate several different downstream kinases including the non-receptor tyrosine kinase Src and the serine/threonine kinase Akt[138], to activate NF-κB. These GTPases have also been shown to activate the transcription factor activator protein 1 (AP-1) downstream of RAGE[138], and to activate phosphatidylinositol 3-kinase (PI3K)[138], which cause NF-κB independent effects. Because activation of these GTPases incites rearrangements of the actin cytoskeleton, RAGE stimulation has been implicated in cellular remodeling events such as migration[137,139], neurite outgrowth[136], and adhesion[140], which play important roles in both homeostasis and immunity. Outside of the Rho family, the related GTPase Ras is also activated downstream of RAGE[141]. It is not entirely clear if this activation results directly from RAGE or from an increase in oxidant stress caused by other pathways activated by RAGE. Ras downstream of RAGE leads to NF-κB p65 activation via the intermediate kinases MEK1 and p42/44 ERK[141,142]. There is also evidence that RAGE may be able to activate ERK and other kinases such as p38 MAPK[142,143] and JAK2[144] directly, without intervening G proteins. The mechanisms by which this could occur are not entirely clear, but ERK has been reported to induce NF-κB activation via phosphorylation of IκB kinase α/β (IKKα/β)[145] and to interact directly with the cytosolic tail region of

RAGE[146]. A cytosolic formin-homology domain-containing adaptor molecule, Diaphanous-1, also appears to directly interact with the RAGE cytosolic tail, and this interaction is essential for RAGE-dependent activation of Rho family GTPases[137]. Because Diaphanous-1 is known to interact with proteins that bind to the actin cytoskeleton[137], this lends further evidence for a direct role of RAGE signaling in cellular remodeling. Much remains to be clarified regarding how these pathways intersect functionally in distinct RAGE-expressing cell types. What is thus far clear is that both overtly immune effects such as cytokine production as well as more general effects such as cell growth and motility are outcomes of RAGE:ligand interactions.

While a close association between RAGE signaling cascades and the cytoskeleton suggest that RAGE is likely to traffic upon ligation of activating signals, the precise cell biology of this process is incompletely described. It has been shown that receptor internalization is an essential event for ERK activation downstream of RAGE in a neuroblastoma cell line[147]. Additional insight comes from the observation that RAGE may interact closely with the structural component of membrane caveolae, caveolin-1, and that integrity of caveolae is required for both NF-κB and JAK-STAT activation by RAGE ligands in vascular smooth muscle cells[148]. Collectively, these observations suggest that RAGE engages with extracellular ligands on the cell surface and mediates their uptake, via caveolae, into intracellular compartments, from which downstream signals can occur.

Expression in tissues and cells: complexity and ties to disease

The variety of RAGE signals and downstream functions take on an additional level of complexity when one considers the diversity of cell types in which RAGE is expressed. Studies of RAGE expression patterns in mammals suggest that there is very low, but constitutive, expression in most organs. This seems to be attributable to expression by vascular endothelial and smooth muscle cells, which infiltrate all body tissues. Unlike other adult tissues that have very little RAGE, the mammalian lung shows strong constitutive levels of the receptor protein, which has been attributed to its expression primarily on type-I alveolar epithelial cells[140,149]. RAGE has been reported to be expressed on a number of leukocytes[150-153], though it is not clear if this expression is constitutive. Nevertheless, the signaling pathways shown to be activated by RAGE are common in many immune cell types, and such cells are often present at sites of accumulation of RAGE ligands, particularly HMGB1 and S100 proteins. Because leukocyte functions are closely associated with the endothelia and epithelia through which such cells extravasate, distinguishing between RAGE-mediated effects from leukocytes versus ones originating from structural cells *in vivo* is an interesting, important, and largely unexplored question. Reductionist studies of cell lines *in vitro* have yielded important insights into signaling pathways but stop short of assessing interactions between multiple cell types. Conversely, disease models *in vivo* have indicated important RAGE-dependent phenotypes, but often yield little insight into the full interplay of mechanisms that lead to them. Hence,

the tissue site of RAGE:ligand interaction is an open and intriguing question. The immune/structural cell interplay is particularly interesting in light of the implication of RAGE in many pathologies that involve inflammation resulting in alteration of tissue structure, or vice versa. These include lung diseases such as fibrosis, cancer, acute respiratory distress syndrome and certain infections[149]. RAGE has also been associated with diseases of the vasculature that have an inflammatory component, including atherosclerosis, diabetic nephropathy and Alzheimer's disease. Animal models have implicated RAGE in a range of other inflammatory conditions including colitis, arthritis, microbial sepsis, and autoimmune syndromes such as experimental autoimmune encephalitis and multiple sclerosis[154,155]. These studies provide strong evidence for the importance of RAGE in inflammatory syndromes and the need to identify and untangle the web of signals across cell types and tissues that RAGE may effect upon recognizing its array of ligands.

Thesis rationale, objectives, and summary

Nucleic acid sensing is currently a vibrant subfield of innate immunity research. Our laboratory has previously focused on the molecular and cellular biology of TLR9 and its oligonucleotide ligands. The mechanisms by which nucleic acid ligands placed into cell culture medium or injected into animals (*i.e.*, extracellular nucleic acids) are taken up into TLR-containing intracellular compartments have

been a long-standing and largely unsolved point of interest. In this context, and in the course of collaborative work on HMGB1, we discovered that the cell surface receptor RAGE could bind directly to TLR9-stimulatory oligonucleotides*. The research presented in this thesis was designed to evaluate the role of RAGE in uptake and presentation of nucleic acids to endosomal receptors, as well as potential independent roles for RAGE as a nucleic acid sensor. Chapter II presents extensive research into the chemical nature of nucleic acid ligands that bind to RAGE and the oligomeric nature of the receptor complex, the biology of RAGE:ligand uptake, and the manner in which RAGE enhances TLR9-mediated inflammatory responses to CpG DNA both *in vitro* and *in vivo*. In physiological contexts, DNA is closely associated with another RAGE ligand, HMGB1, and the ways in which these two ligands and other multimolecular complexes interact with RAGE is explored in Chapter III. Chapter IV considers how the data presented herein relate to previous knowledge about RAGE and other immune receptors, as well as implications for understanding larger questions of immunity and disease, and questions yet to be addressed.

This work contributes to our understanding of the role of RAGE in modulating immune responses, particularly those initiated by nucleic acids. As such, it provides an important link in conceptualizing how RAGE contributes to immune complex-forming autoimmune diseases and inflammatory pathologies of

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** At the time, DNA as a RAGE ligand was not yet mentioned in the scientific literature.*

the lung, as well as designing precise biopharmaceutical therapeutic agents for their treatment and prevention.

PREFACE to Chapter II

A modified version of this chapter is under review for publication:

Sirois CM, Jin T, Miller AL, Nakamura H, Horvath GL, Mian A, Jiang J, Schrum J, Brewah Y, Tian J, Chang C-S, Chowdhury PS, Sims GP, Kolbeck R, Coyle AJ, Humbles AA, Xiao TS, Latz E. **RAGE is a nucleic acid receptor that promotes inflammatory responses to DNA.**

This chapter represents the main thesis project of C.M.S, who generated all data except that indicated below. C.M.S. created all of the figures from primary data with the exception of Figure 2.6 (by T.S.X.) and Figure 2.8 (by E.L.), and was the principal writer and coordinator of the manuscript text.

Co-authors contributed the following data:

T.J., A.M., J.J., T.S.X.: Crystallography data in Figure 2.3b-c, Figure 2.4, Figure 2.5a, Figure 2.6, and Table 2.1.

E.L., Figure 2.3e (based on preliminary studies by C.M.S.).

H.N, J.S., E.L., Figure 2.5 (2.5a based on preliminary studies by C.M.S.)

A.L.M, Y.B., A.A.H.: *In vivo* data in Figure 2.9 (experiments designed in collaboration with C.M.S. and E.L.; statistical analysis by C.M.S.).

G.L.H. aided in raw data analysis for Figure 2.5d.

Figure 2.10 was designed by Mariane Bandeira de Melo.

CHAPTER II: RAGE recognizes nucleic acids and promotes inflammatory responses to DNA

Abstract

Nucleic acids are sensed by the immune system during infections and tissue damage. Activation of the endosome-resident signaling receptors is critically limited by restricting the access of nucleic acids from the extracellular space. Here we show that the receptor for advanced glycation end-products (RAGE) promoted DNA uptake into the endosomal network and lowered the threshold for activation by DNA. Structural analysis indicated that DNA interacted with dimers of the outermost RAGE extracellular domains, and could induce formation of higher-order RAGE complexes. Mice deficient in RAGE were unable to mount a typical inflammatory response to CpG DNA in the lung, indicating that RAGE is a key component in fine-tuning interaction of nucleic acids with their sensors.

Introduction

Infection and tissue damage cause an immediate inflammatory response that is characterized by the activation of local structural cells and innate immune cells and by rapid recruitment of additional immune cells to the affected site. This response serves to control the invading pathogen and to initiate reparative processes that restore tissue function. However, the inflammatory process must be well balanced, as an exaggerated immune response may result in collateral tissue damage.

In the last decade, much has been learned about the molecular mechanisms that orchestrate inflammatory responses. The immune system becomes activated when any of a diverse array of receptor proteins engages its respective ligand molecule(s). These ligands may originate either from an infectious microbial source or from the host itself, under conditions of cell stress and tissue damage. Under normal homeostatic conditions, such immune triggers are absent or scarce in the host or are sequestered so as to prevent access to compartments containing signaling receptors. Immunity-triggering molecules take a number of distinct chemical forms, which are recognized by several families of germline-encoded innate immune signaling receptors expressed either on membranes[156] or in the cytosol[157]. Certain surface-exposed immune receptors recognize a number of lipidated or proteinaceous activators that are foreign to the host, such as lipopeptides (Toll-like receptor (TLR)2),

lipopolysaccharides (TLR4), and flagellin (TLR5). In contrast, many signaling receptors do not have immediate access to the extracellular space, as they are expressed in the endoplasmic reticulum and endosomal membranes (TLR3, 7, 8 and 9) or in the cytosol (RIG-I like receptors, AIM-2, and as-yet undefined DNA sensors). Indeed, all described immune sensors devoted to the recognition of nucleic acids are expressed in compartments that are sequestered away from the extracellular milieu.

Nucleic acids present a unique challenge to the immune system: on one hand, sensing of microbial genetic materials is an efficient way to detect viruses and other types of invading pathogens. However, mechanisms must exist for distinguishing such exogenous nucleic acids from the host's own DNA and RNA. Sequestration of nucleic acid sensing receptors has been proposed to limit receptor triggering by self nucleic acids that are present in the extracellular space under homeostatic conditions, presumably due to normal cell turnover. Indeed, experimental mis-localization of TLR9, an endosomal receptor for DNA, to the plasma membrane led to the recognition of self-DNA from the extracellular environment[42]. At the same time, endosomal localization of TLR9 was required for efficient recognition of viral DNA[42], suggesting that nucleic acids become concentrated in endosomal compartments or that additional mechanisms of receptor processing are required for nucleic acid recognition in the endosome[45,158,159].

Along with compartmentalization, other safeguards typically prevent self nucleic acid recognition by innate immune receptors. For example, nucleases present in the extracellular space (DNAse I), in endosomal compartments (DNAse II) and in the cytosol (DNAse III) digest DNA and ensure that DNA released under normal conditions escapes detection by nucleic acid sensors[160-164]. However, if nucleic acid concentrations exceed the nuclease capacity, such as during infections or in situations of increased cell damage, signaling receptors and their downstream inflammatory effects can be triggered. Chronic activation of inflammatory responses by nucleic acids can result in undesirable autoimmune syndromes and dramatic pathologies[164,165], yet the recognition of DNA during tissue destruction is an integral part of the host immune and repair responses[166]. Hence, effective management of self-tolerance and damage sensing appears to require the coordinated delivery of extracellular DNA to intracellular sites of recognition.

Here, we identify the receptor for advanced glycation end-products (RAGE) as a cell surface receptor for nucleic acids. RAGE binds directly to DNA and RNA and promotes their uptake into endosomal compartments, thereby sensitizing cells to extracellular nucleic acids. A co-crystal structure of RAGE with DNA supports the concept that RAGE binds to nucleic acids via interaction with the charged sugar-phosphate backbones in a sequence-independent manner. RAGE deficiency largely prevents an inflammatory response towards TLR9

stimulatory DNA in the lungs, suggesting that RAGE plays an important role for the recognition of DNA in vivo.

Results

RAGE concentrates DNA on cells and interacts with DNA in a sequence-independent manner.

To address the role of RAGE in cellular responses to DNA, we generated cell lines expressing chimeric fluorescent RAGE driven by a tetracycline-inducible promoter. Uninduced cells did not show significant RAGE expression and we observed only a low amount of DNA binding to the cells (**Figure 2.1a**). RAGE-mCitrine expression was approximately 100-fold higher after promoter induction and RAGE-expressing cells bound 10- to 100-fold more fluorescently labeled DNA compared to uninduced cells (**Figure 2.1a**). In addition, the amount of DNA binding correlated directly with the level of RAGE expression (**Figure 2.1a**). Notably, when incubated with fluorescently labeled DNA and visualized by confocal microscopy, cells expressing RAGE had visibly more DNA bound at the cell surface than cells not expressing RAGE (**Figure 2.1b**). These data suggested that RAGE interacts with and concentrates DNA on cell surfaces.

We next sought to identify the characteristics of RAGE-binding nucleic acids. We found that several structural classes of CpG oligonucleotides, which are known to activate TLR9[167], bound to purified RAGE protein with low

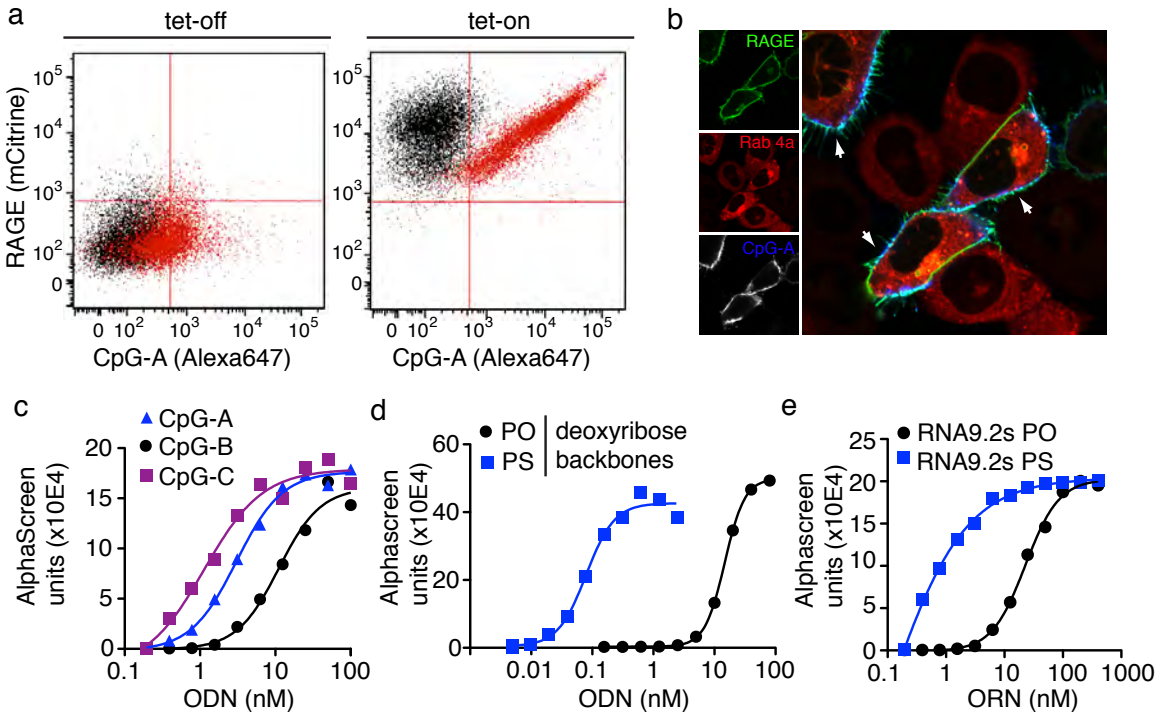

Figure 2.1 RAGE binds directly to nucleic acid ligands on the cell surface. (**a**) 293T cells expressing tetracycline-inducible RAGE-mCitrine were left uninduced (left panel, black population) or treated to induce RAGE expression (right panel, black population) and then incubated on ice with 1 μM AlexaFluor 647-labeled ODN 2336 (CpG-A, red populations), washed, and analyzed by flow cytometry. (**b**) HeLa cells expressing Rab 4a-YFP (red) to visualize the cytoplasm, with or without coexpression of RAGE-YFP (green), were incubated with 1 μM AlexaFluor 647-labeled ODN 2336 (CpG-A, blue; white in small panel for better viewing) for 5 minutes. Excess DNA was washed away and live cells were imaged by confocal microscopy. White arrows indicate RAGE-positive cells exhibiting DNA binding at the cell surface. (**c**) Ability of purified, his-tagged human RAGE extracellular domain to bind to biotinylated oligonucleotide ligands in solution was assessed by AlphaScreen homogenous binding assay. Indicated concentrations of oligodeoxynucleotides (ODN) or oligoribonucleotides (ORN) were incubated with 40 nM RAGE (V-C1-C2)-his. PO, phosphodiester-linked deoxyribose backbone; PS, phosphorothioate-linked deoxyribose backbone.

nanomolar affinities (**Figure 2.1c**). However, in contrast with previous findings[128], oligodeoxynucleotides (ODN) bound RAGE in a sequence-independent manner (**Figure 2.2a**) and even phosphodiester and phosphorothioate sugar-phosphate backbones without nucleotide bases bound to RAGE with apparent high affinities (**Figure 2.1d**). We observed similar binding affinities for single-stranded (ss) and double-stranded (ds) forms of DNA (**Figure 2.2b**) and RAGE was able to bind ODN as short as 15 bases in length (**Figure 2.2c**). In addition, we found that RNA could interact with RAGE at apparent high affinity (**Figure 2.1e**). These studies indicated that nucleotide bases play a minimal role in DNA binding and that RAGE may bind to nucleic acids by interaction with the sugar-phosphate backbones.

RAGE binds DNA through electrostatic attractions with the backbone

The RAGE extracellular region is composed of three structural domains named according to their homology to other members of the immunoglobulin superfamily: an N-terminal variable-type (V) (or I-type, refer to [111]) domain, followed by two membrane-proximal constant-type domains (C1 and C2). The V and C1 domains are thought to form a single structural unit, which is separated from the C2 domain by a flexible linker region[168]. To assess which part of RAGE interacts with DNA, we investigated DNA binding to recombinant V domain, V-C1 domains or C1-C2 domains, respectively. DNA binding was mediated primarily by the outermost V domain of RAGE (**Figure 2.3a**), similar to what has been

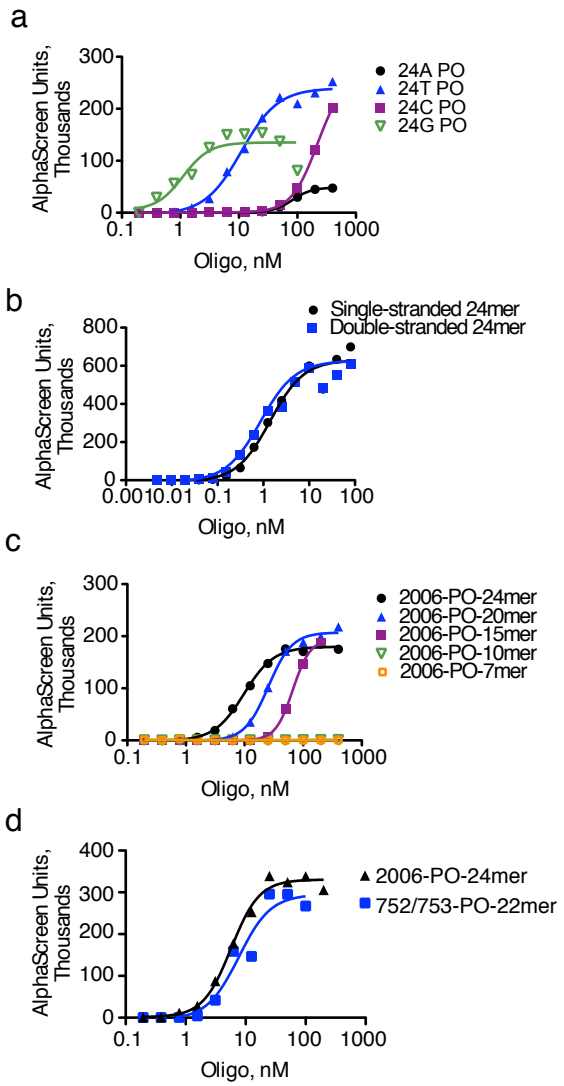

Figure 2.2 Additional binding data: RAGE binds directly to oligodeoxy-nucleotides of distinct sequences and sizes.
Indicated concentrations of ODN were incubated with 40 nM his-tagged RAGE V-C1-C2 and binding was assessed by AlphaScreen. Binding was tested for (a) homopolymers consisting of 24 nucleotides of adenosine (A), thymine (T), cytosine (C) or guanine (G) with a phosphodiester backbone (PO); (b) single-stranded 24-mer ODN 2006 with a phosphodiester backbone (black circles) and the same oligo annealed to its complementary strand (blue squares); (c) full length ODN 2006 with a phosphodiester backbone (2006-PO-24mer) and with forms of the same ODN truncated from the 3' end; (d) the double-stranded phosphodiester-backbone vaccinia virus-derived DNA sequence (752/753-PO-22mer) used in the co-crystal. Single stranded 2006-PO-24mer is shown for reference.

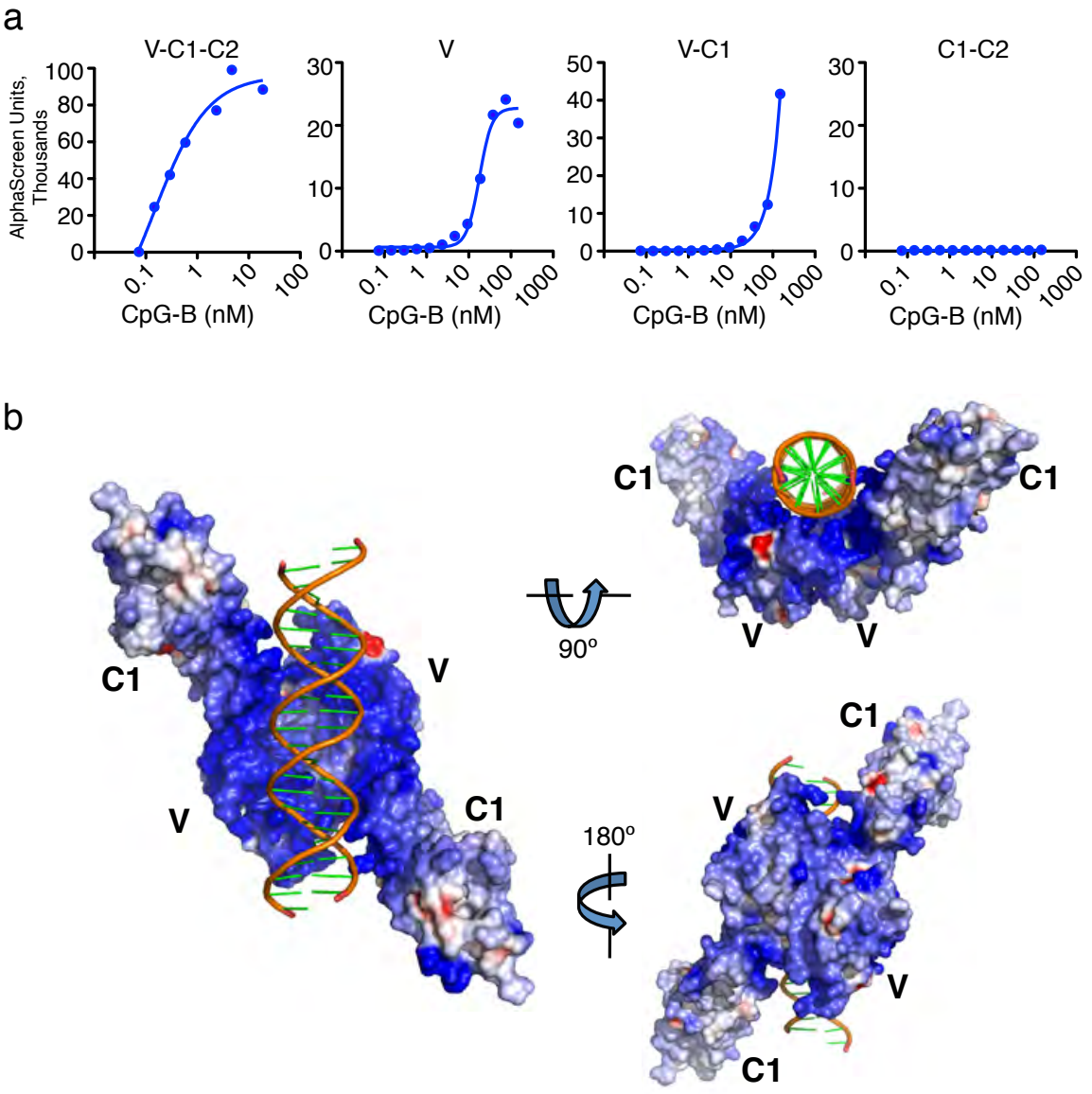

Figure 2.3a-b DNA interacts with a region of positive charges at the RAGE dimer interface.

(**a**) Binding in solution of biotinylated ODN 2006 (CpG-B) to full length human RAGE extracellular domain (V-C1-C2-his) or isolated structural domains was evaluated by AlphaScreen homogenous binding assay. Indicated concentrations of CpG-B were incubated with 40 nM RAGE. (**b**) Electrostatic charge surface of RAGE is shown on a scale of -10 kT/e (red) to +10 kT/e (blue) in three different orientations. The bound dsDNA is shown as an orange ribbon.

Figure 2.3c-d DNA interacts with a region of positive charges at the RAGE dimer interface. (**c**) Surface representation (center) showing positively charged residues lining the RAGE dimer interface where DNA binding occurs. DNA-binding residues are colored green on one RAGE molecule ("molecule A" in the text) and cyan on the other ("molecule B" in the text). Side panels show the molecular details of the two discrete binding sites, indicated by red boxes, for each RAGE V-C1 domain colored green and cyan. Hydrogen bonds are indicated with dotted lines, and the two strands of the dsDNA are colored silver and yellow, respectively. (**d**) Polyethyleneimine (PEI) competes with RAGE for binding to CpG-B (PO and PS backbones), CpG-A and CpG-C.

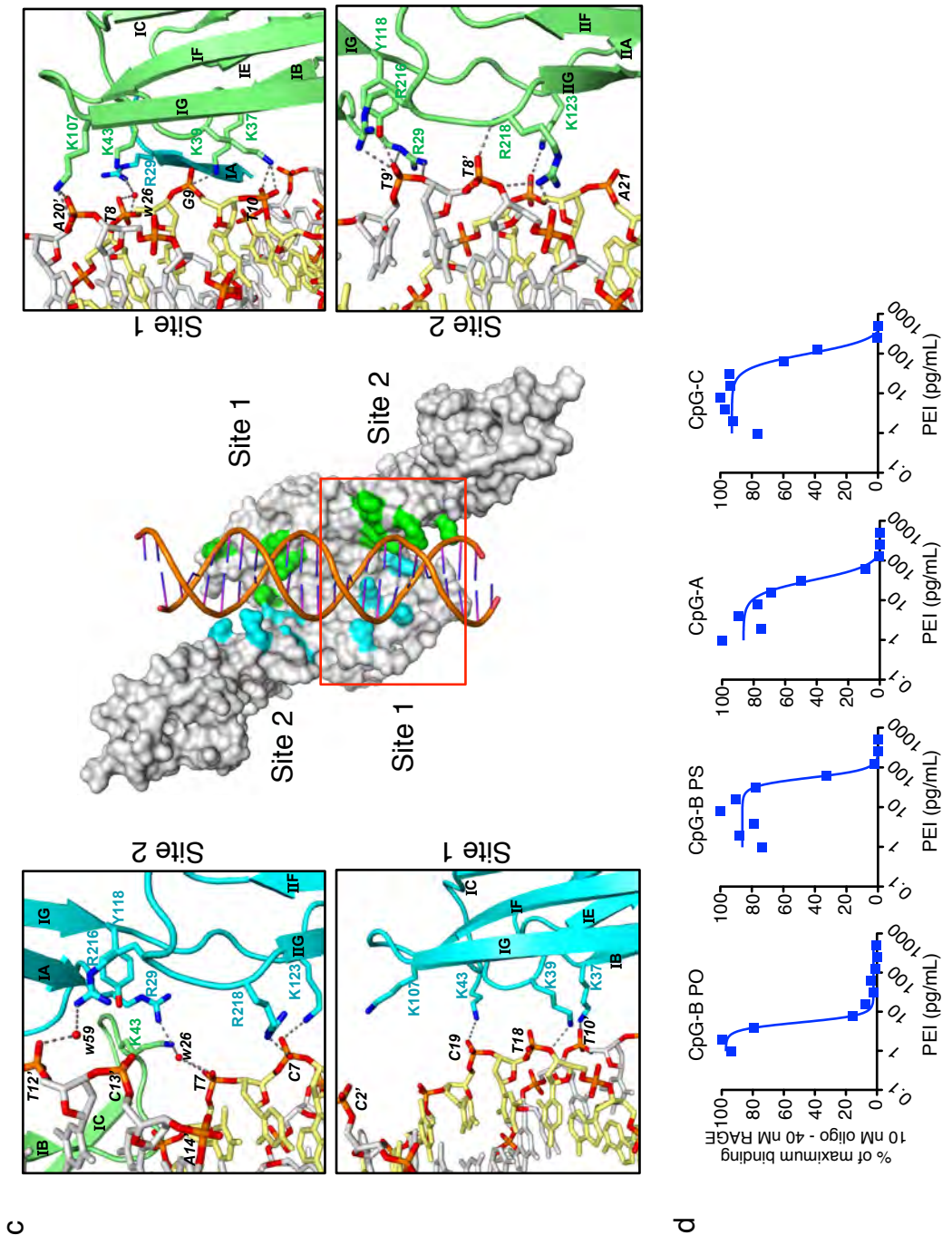

Figure 2.3c-d DNA interacts with a region of positive charges at the RAGE dimer interface.
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reported for binding of other RAGE ligands[129,168,169]. The C2 domain did not directly bind DNA in our assays (**Figure 2.3a** and data not shown), but may enhance ligand binding by promoting proper folding and charge relationships throughout the extracellular domain, as DNA binding to V-C1-C2 appears to be more favorable than to V-C1 alone.

To understand the molecular details of the RAGE:DNA interaction, we determined the crystal structures of the RAGE V-C1 domain in complex with a 22 nucleotide dsDNA molecule derived from vaccinia virus genomic repeat sequences and a 22mer CpG motif-containing dsDNA molecule, at resolutions of 2.8 Å and 3.1 Å, respectively (**Table 2.1** and **Figure 2.4a**). As the structures were essentially the same except for the two dsDNA sequences, the following discussion will focus on the higher resolution 2.8 Å structure. The structures were solved by molecular replacement using two previously published RAGE V-C1 domain structures (PDB accession numbers 3CJJ[111] and 3O3U[129]) as the search models. The structures revealed that the RAGE V-C1 domains formed a homodimer with the dsDNA bound in a concave cradle near the dimer interface (**Figure 2.3b**). The two V-C1 monomers interacted with each other in a dyad configuration with their V domains located at the dimer interface (see below), creating an extensive positively charged pocket where dsDNA binds. Excellent charge and shape complementarity were observed between the RAGE interface and the bound dsDNA. Close examination of the protein:DNA interaction surface

Table 2.1 X-ray crystallography data collection and refinement statistics.

	I (752-753, VV)	II (981-982, CpG)
Data Collection		
Spacegroup	$P6_1$	$P6_1$
Unit cell (a, b, c) (Å)	79.12, 79.12, 224.04	77.92, 77.92, 224.39
(α, β, γ) (°)	90, 90, 120	90, 90, 120
Wavelength (Å)	1.00	1.00
Resolution (last shell) (Å)	50-2.80 (2.85-2.80)	50-3.10 (3.15-3.10)
No of reflections (total/ unique)	154198/19330	138765/13817
Completeness (last shell) (%)	99.4 (96.6) *	99.0 (85.6) *
I/s(I) (last shell)	14.49 (1.97) *	16.10 (1.97) *
Rmerge (last shell) (%)¶	10.7 (60.7) *	14.0 (65.3) *
Refinement		
Number of protein atoms	3267	3274
No. of DNA base pairs	22	22
No. of solvent/hetero-atoms	75	61
Rmsd bond lengths (Å)	0.007	0.008
Rmsd bond angles (°)	0.982	0.975
Rwork (%)†	19.6	19.1
Rfree (%)‡	23.8	23.1
Ramachandran plot favored/disallowed (%) **	95.7/0	95.7/0
PDB accession code	3S59	3S58

*Asterisked numbers correspond to the last resolution shell.

¶ $R_{merge} = \sum_h \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ and $\langle I(h) \rangle$ are the ith and mean measurement of the intensity of reflection *h*.

† $R_{work} = \sum_h ||F_{obs}(h)| - |F_{calc}(h)|| / \sum_h |F_{obs}(h)|$, where $F_{obs}(h)$ and $F_{calc}(h)$ are the observed and calculated structure factors, respectively. No I/σ cutoff was applied.

‡ R_{free} is the R value obtained for a test set of reflections consisting of a randomly selected 5% subset of the data set excluded from refinement.

**Values from Molprobity server (http://molprobity.biochem.duke.edu/)

Figure 2.4 Additional data: RAGE binds dsDNA at the dimer interface.
(**a**) On the left, refined 2*Fo-Fc* map for the dsDNA is shown as magenta mesh superimposed on the refined model. On the right, the top 10 molecular replacement solutions for the dsDNA are shown as orange ribbons, with the refined dsDNA in red ribbon. (**b**) Footprint of the RAGE dimer on the dsDNA colored cyan and green for each of the V-C1 domains. (**c**) The two RAGE V-C1 molecules (cyan and green) in the crystal are superimposed with their bound dsDNA (blue and green), showing the switched positions for the major and minor grooves of the dsDNA in reference to the V-C1 domains. (**d**) The RAGE dimer interface centered at the V domain. Residues involved in RAGE dimerization are shown as sticks and colored cyan and green as the respective molecules. Water molecules are shown as red spheres and hydrogen bonds as dotted lines. The crystallographic dimer from 3CJJ is superimposed in our DNA-bound RAGE dimer structure in wheat and orange.

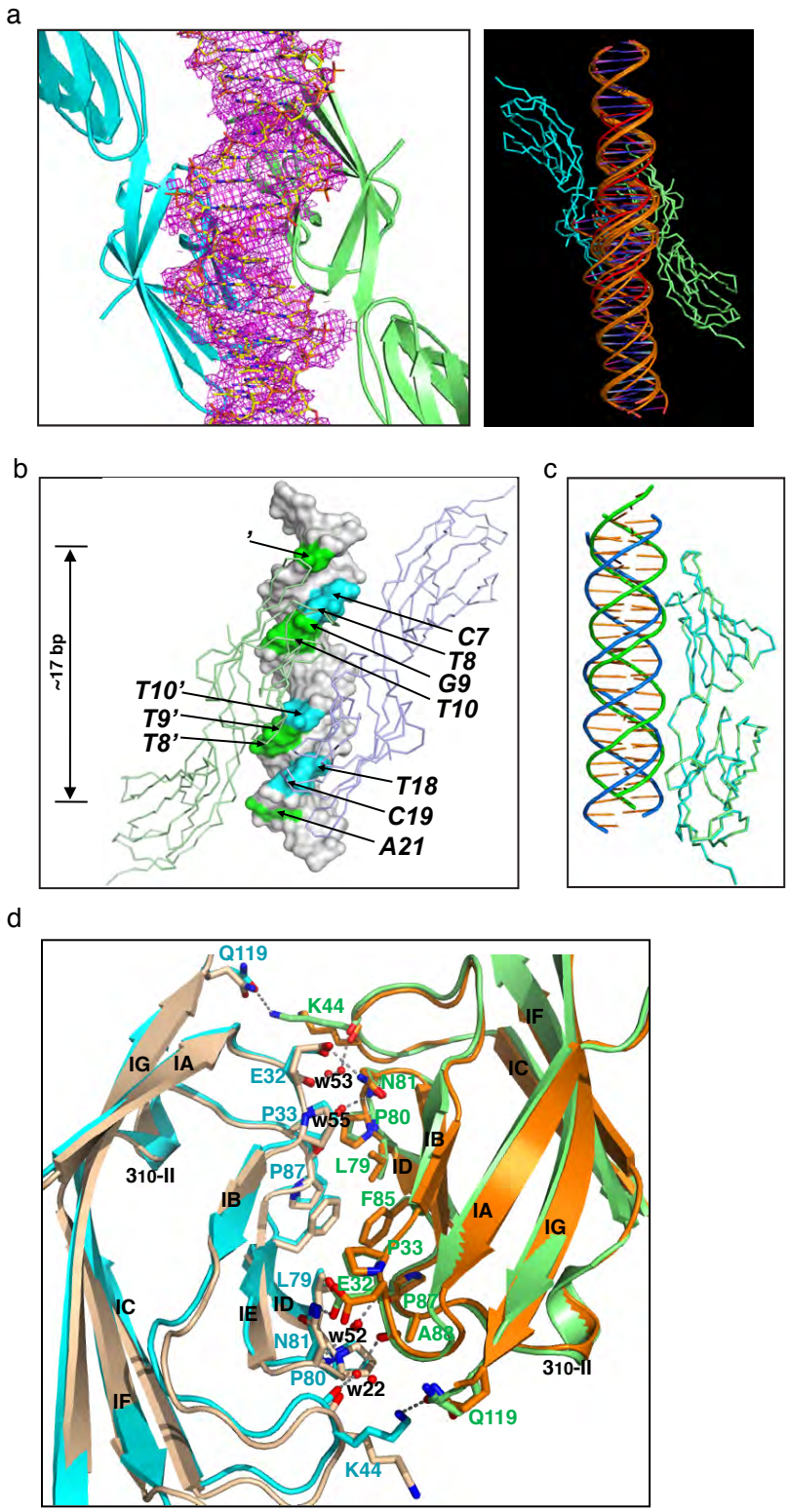

Figure 2.4 Additional data: RAGE binds dsDNA at the dimer interface.

indicated that ~7 positively charged residues on each V-C1 unit were in close proximity to the negatively charged DNA backbone, spanning ~17 bp (**Figure 2.3c** and **Figure 2.4b**), in agreement with our finding that the minimal length requirement for DNA binding was approximately 15 bases (**Figure 2.2c**). Each of the RAGE V-C1 domains engage both strands of the dsDNA through basic or hydrophilic residues from RAGE interacting with oxygen atoms of the DNA backbone and, in some cases, through coordinating water molecules.

A total of 1300 $Å^2$ of solvent accessible surface area was buried at the RAGE:DNA interface. There are two DNA binding patches on each RAGE molecule, hereafter referred to as “site 1” and “site 2” (**Figure 2.3c**). Site 1 is located entirely in the V domain near β-strand B (βB) and the connecting loops containing residues K37, K39 and K43, as well as K107 from β-strand G. Notably, residues K43 from molecule A and R29 from molecule B coordinate the same water molecule (w26) that forms a hydrogen bond with an oxygen of the DNA phosphate backbone (**Figure 2.3c** upper right panel). Site 2 is located at the juncture of the V and C1 domains, and includes residues R29 at the C-terminus of βA, Y118 and K123 of the V-C1 linker, and R216 and R218 of βG in the C1 domain. The amide group of R218 forms a direct hydrogen bond with a backbone oxygen of DNA (**Figure 2.3c** bottom right panel). The footprints of the two RAGE molecules on the dsDNA are very similar but not identical (**Figure 2.4c**), with the dsDNA switching its location of the major and minor grooves relative to the RAGE molecules, suggesting a rather flexible surface of the RAGE

dimer that may be able to accommodate various forms of nucleic acids such as ds or ss DNA and RNA. This is consistent with the dominant presence of flexible lysine and arginine residues at the DNA binding surface.

In contrast to sequence-specific DNA recognition by proteins such as transcription factors, the current structures showed no contact between RAGE and the nucleotide bases. Because the overall negative charge on the DNA molecule is determined by its phosphate backbone, we tested whether cationic polymer polyethyleneimine (PEI) could compete with RAGE for DNA binding. Our results showed that PEI could "mask" the negative charge of DNA and was sufficient to negate DNA:RAGE binding *in vitro* (**Figure 2.3d**), further highlighting the electrostatic nature of the recognition.

No major conformational changes were observed in the RAGE V-C1 domains between the DNA-bound structures and those of the non-DNA bound forms (3CJJ[129] and 3O3U[111]; **Figure 2.5a**), consistent with the observation that the linkage between the V and C1 domains is fairly rigid[168] and is not modified by the binding of DNA ligands.

Dimerization of the RAGE V-C1 domains

A hydrophobic surface on the V domain is located at the center of the RAGE dimer interface in the current configuration we call "trans." It is composed of residues L79, P80, F85, P87, and A88 from the βE strand and the connecting loops, plus P33 and V35 from the βB strand, and P45 and P46 from the βB-βC

loop (**Figure 2.4d** and **Figure 2.5a**). The hydrophobic patch is surrounded by hydrophilic residues decorating the circumference of the dimer interface. A previous structure of RAGE V-C1 in the absence of bound DNA showed a crystallographic C1-C1 dimer that we call “cis,” which is centered at a bound zinc ion[111]. In comparison with the V domain-centered trans dimer interface, the cis dimer interface is hydrophilic and smaller in size. Unexpectedly, examination of the 3CJJ crystal lattice packing revealed that the trans dimers are also present in this crystal (**Figure 2.5a**), though the DNA binding site is occluded by crystallographic symmetry mates. It is thus clear that the trans dimer formation is independent of DNA binding, and both the trans and the cis dimers can co-exist as in the 3CJJ crystal. This is consistent with our observation that the most intense fluorescence of RAGE on the cell surface is located at the juncture of two adjacent cells (**Figure 2.5e**, **Figure 2.8c**), indicating that both trans and cis dimerization may be involved in the formation of the RAGE oligomers, with the cis and trans configurations mediating RAGE interaction on the same cell and adjacent cells, respectively.

The chemical nature of the trans dimerization interface, including a typical buried solvent-accessible surface area of over 1000 $Å^2$, together with the observations that RAGE V[170], V-C1[19], or V-C1-C2[171] domains spontaneously dimerize in solution, suggests that the V-domain mediated dimers are likely to be present under physiological conditions. As RAGE molecules are highly

Figure 2.5 Constitutive RAGE dimers form higher order oligomers in the presence of complex DNA ligands. (**a**) Ribbon diagrams of the RAGE V-C1 dimerization interface. Left, RAGE V-C1 crystal packing in the non-DNA bound form (3CJJ)[1], with V-C1 monomers colored yellow, orange, and wheat. The orange sphere indicates a zinc ion. Middle, wheat and orange V-C1 domains from the left superimposed onto the V-C1 domains (cyan and green) in the current DNA-bound crystal structure. The dimer interface at the V domain (red circle) is represented in gray surface (right), with the hydrophobic and hydrophilic residues colored orange and green, respectively. (**b**) HEK293 cells expressing RAGE-mCitrine were incubated with or without the amine-reactive crosslinking agent BS3 prior to immunoblot for the mCitrine tag. (**c**) HEK293 cells co-expressing RAGE-CFP and RAGE-YFP were analyzed for FRET by flow cytometry. Cells show detectable baseline FRET (black histogram) in the absence of ligand. Cells expressing only RAGE-CFP (FRET-negative, gray histogram) are shown for reference. (**d**) Cells from (c) were incubated with the indicated concentrations of oligonucleotides and analyzed for FRET by flow cytometry. Graphs show the percent increase in FRET signal over baseline FRET. “x2” indicates biotinylated ODN complexed with streptavidin in a 2:1 ratio. (**e**) HEK293 cells co-expressing RAGE-mCerulean and RAGE-mCitrine were incubated for 30 minutes at 37 ºC with AlexaFluor 647-labeled CpG-A or CpG-B and analyzed for FRET by confocal microscopy. FRET efficiency in individual cells is plotted, with lines indicating the mean value for each condition.

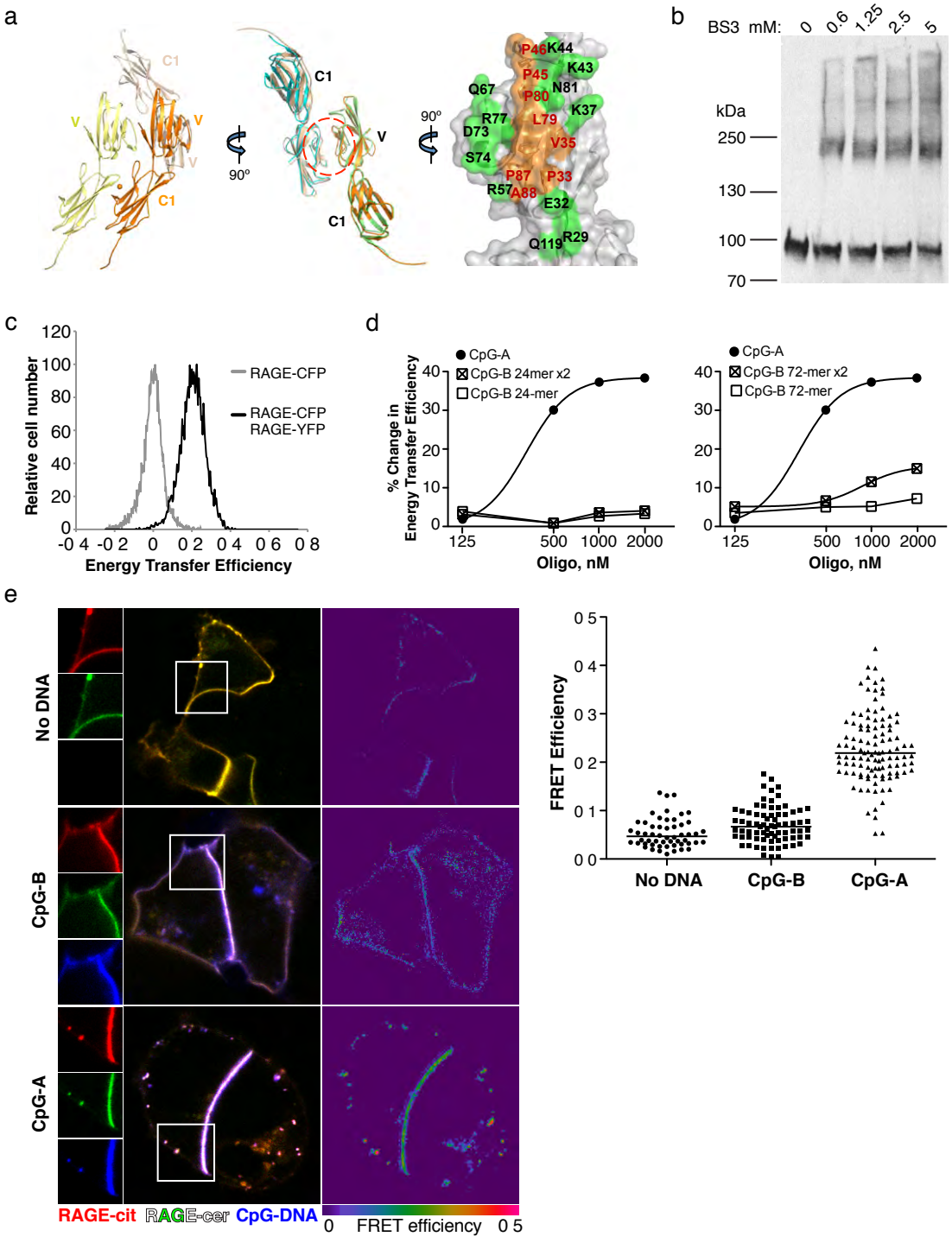

Figure 2.5 Constitutive RAGE dimers form higher order oligomers in the presence of complex DNA ligands.

conserved in both their DNA binding sites and dimerization sites (**Figure 2.6**), RAGE dimer binding to nucleic acids through electrostatic attraction may be a common mode of its function in different species.

DNA can induce formation of higher-order RAGE oligomers

To confirm RAGE dimer and oligomer formation in live cells, we subjected 293T cells expressing full-length human RAGE-mCitrine to treatment with the cell-impermeable crosslinking agent BS3, followed by cell lysis, protein denaturation and gel electrophoresis. In the presence of crosslinker, higher molecular weight bands corresponding to dimers and higher-order oligomers can be detected in unstimulated cells (**Figure 2.5b**). To further explore the relationship of native RAGE dimers with DNA ligands, we generated dually-labeled cell lines appropriate for Förster resonance energy transfer (FRET) studies. HEK293 cell lines stably expressing RAGE tagged with FRET donor (CFP or mCerulean) and acceptor (YFP or mCitrine) fluorophores were analyzed by both flow cytometry and confocal microscopy for energy transfer between fluorophore tags. A constitutive baseline energy transfer was observed in unstimulated cells (**Figure 2.5c**, **Fig 2.5e**, top panel), supporting the idea that RAGE exists as a dimer in the absence of ligand. Addition of a CpG-B 24-mer oligonucleotide did not alter energy transfer efficiency (**Figure 2.5d**, left panel, **Figure 2.5e**, middle panel). Interestingly, however, the addition of a CpG-A 21-mer induced a dose-dependent increase in FRET efficiency (**Figure 2.5d**, left

Figure 2.6 Sequence alignments of selected RAGE V-C1 domains. The β strands and 310 helices are labeled atop the sequences, with those for V domains noted with “I” and C1 domains “II”. The residues at the trans dimer interface are colored orange and green for hydrophobic and hydrophilic interactions, respectively. The DNA binding residues are shown in red boxes. The two disulfide bonds are shown as “SS1” and “SS2”, respectively.

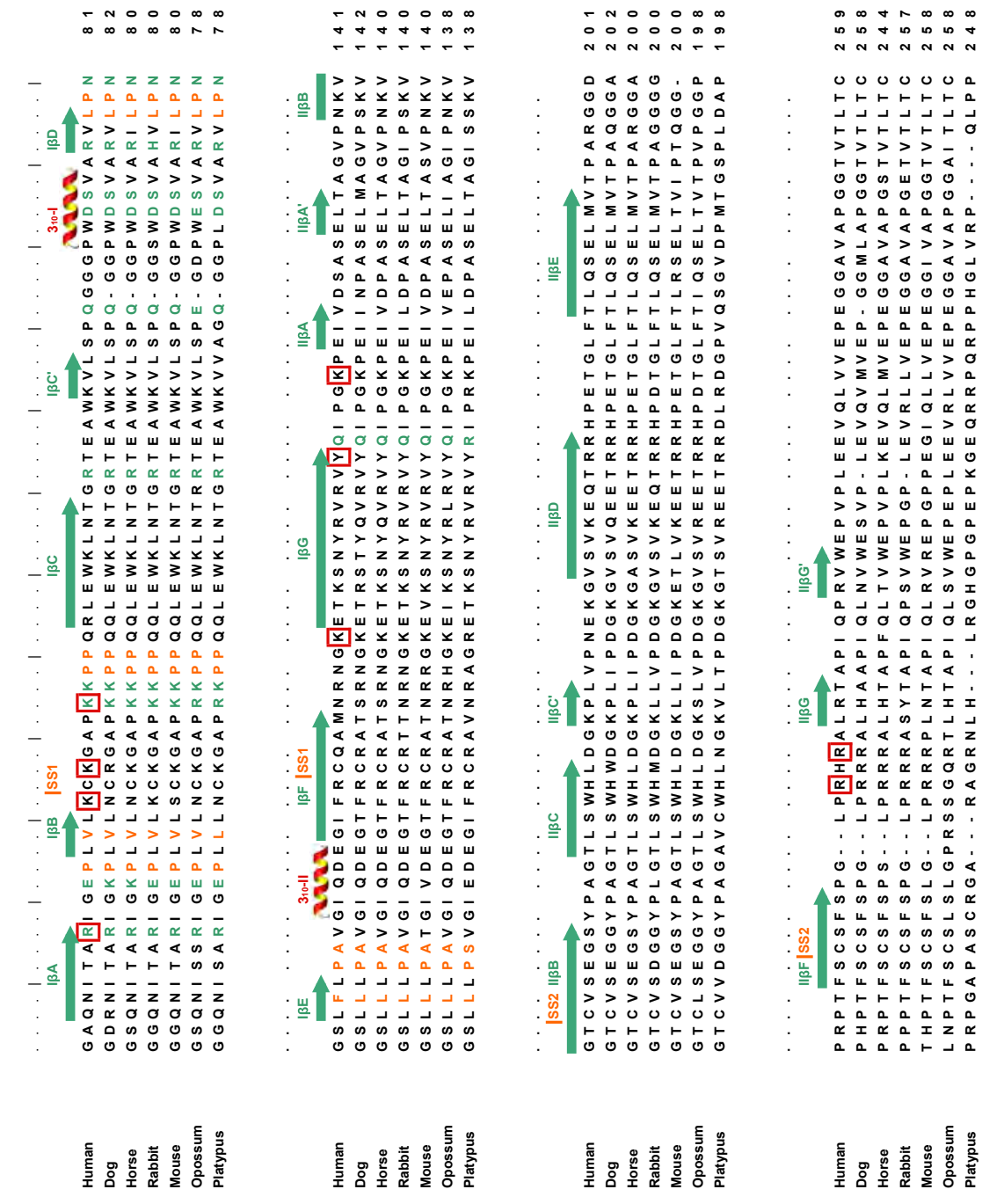

Figure 2.6 Sequence alignments of selected RAGE V-C1 domains.
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panel). CpG-A oligonucleotides are able to adopt large complexes in solution by virtue of G-tetrad formation between poly-G tails on the ends of the ODN[172]. The induced increase in FRET is consistent with higher-order RAGE oligomer formation. In keeping with this observation, CpG-A DNA can be seen to induce aggregation of RAGE into clusters on the cell surface (**Figure 2.5e**, bottom panel), which is not observed in unstimulated cells or cells incubated with CpG-B (**Figure 2.5e**, top and middle panels). Simply increasing the length of the DNA ligand did not promote increased energy transfer (**Figure 2.5d**, right panel), nor did complexation of short, biotinylated ODN sequences with streptavidin (**Figure 2.5d**, left panel). However, biotin-streptavidin complexation of a relatively long ODN increased FRET efficiency (**Figure 2.5d**, right panel). These findings suggest that while RAGE may bind DNA indiscriminately, only large ligand complexes induce receptor reorganization on the cell surface.

RAGE expression promotes DNA uptake by cells via the endosomal route

Having established that RAGE and DNA interact at the cell surface, we sought to understand the functional consequences of this interaction. Cells expressing RAGE not only showed increased binding of both CpG-A and CpG-B DNA to the cell surface, but also a notable increase in total DNA uptake over a 30 minute time period (**Figure 2.7a**), compared to cells not expressing RAGE. This effect was DNA-specific, as binding and uptake of transferrin to its respective receptor (**Figure 2.7a**) or the uptake of TLR2 ligand (not shown) was not

Figure 2.7 RAGE promotes cellular DNA uptake. (**a**) 293T cells expressing tetracycline-inducible RAGE-mCitrine were induced overnight (black bars) or left untreated (white bars) and then incubated with 1μM AlexaFluor 647-labeled transferrin, CpG-A or CpG-B at the indicated temperatures for 30 minutes, washed to remove unbound DNA, and analyzed by flow cytometry. Error bars indicate standard error of the mean. (*) Indicates $p < 0.05$ as assessed by two-tailed t-test. (**b-c**) HeLa cells expressing RAGE-CFP (green) and the early endosome protein Rab 5a-RFP ((b), red) or the late endosome protein Rab 9a-YFP ((c), red) were incubated with 1 μM AlexaFluor 647-labeled CpG-A or CpG-B for 5 minutes, washed to remove unbound DNA, then incubated for the indicated times at 37 ºC prior to analysis by confocal microscopy. Bar graphs show the percentage of Rab-positive endosomes colocalizing with both the RAGE and DNA tags at each time point. Error bars indicate standard error of the mean.

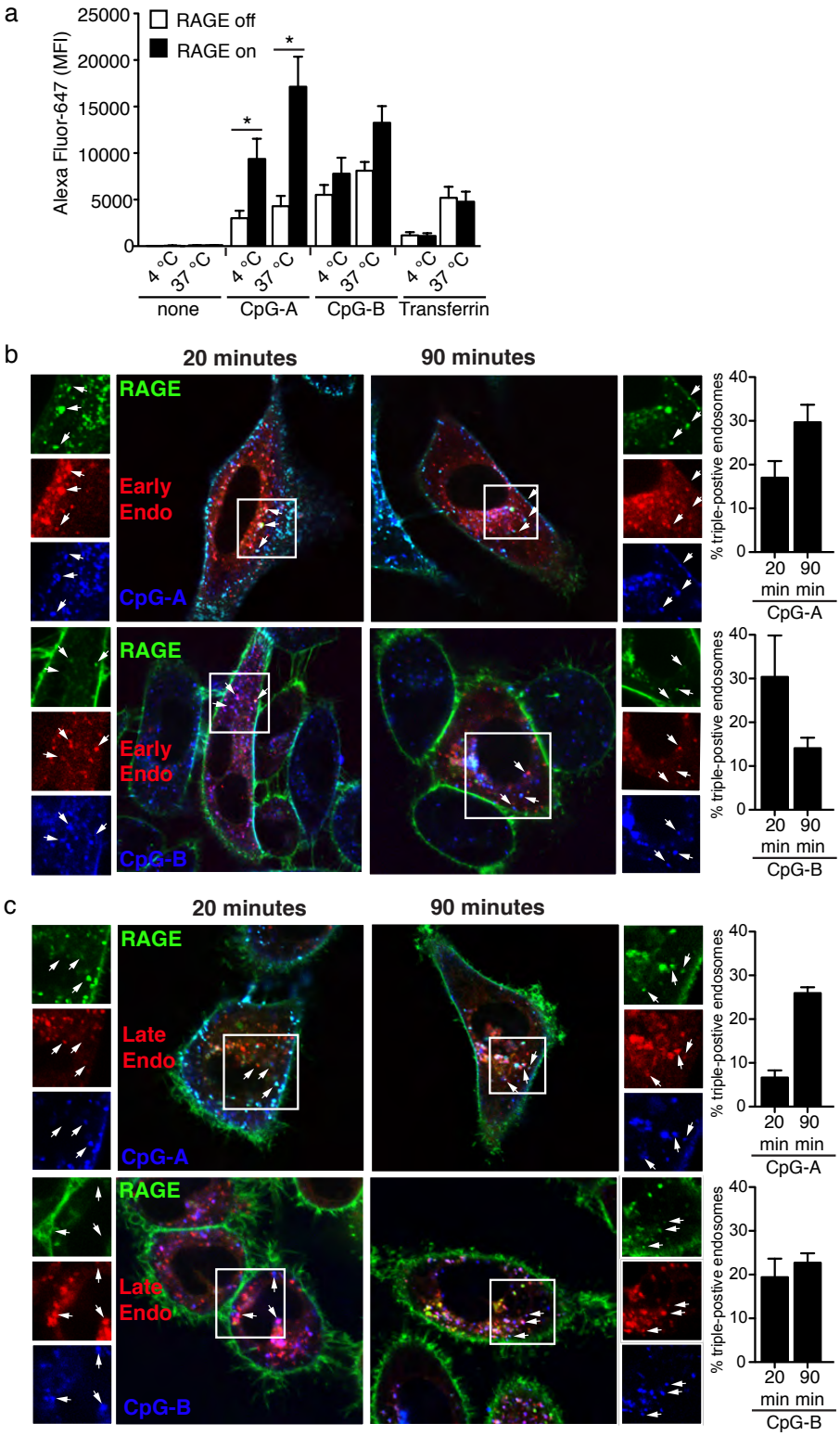


Figure 2.7 RAGE promotes cellular DNA uptake.

influenced by RAGE expression. CpG DNA in cell culture medium gains access to endosomal compartments, where it is able to interact with and stimulate Toll-like receptor 9 (TLR9), although the mechanism by which this uptake occurs is unknown. While cellular uptake of DNA is not strictly RAGE-dependent (Figure 2.7a), we sought to evaluate the nature of the receptor-ligand association after surface binding. We stably expressed fluorescently tagged RAGE together with fluorescently tagged endosomal markers and assessed the binding and trafficking of DNA in live cells. Fluorescently labeled DNA was seen to associate with RAGE at the cell surface and traveled together with the receptor into both early and late endosomal compartments (Figure 2.7b-c). Notably, the kinetics of trafficking differed depending on DNA ligand structure. While CpG-B entered the cell quickly and could be found in late endosomes 20 minutes after exposure, RAGE:CpG-A clusters persisted longer at the cell surface and remained primarily in early endosomes at 20 minutes (Figure 2.7b-c). By 90 minutes, both CpG-A and CpG-B could be seen colocalized with RAGE in late endosomes (Figure 2.7c), the compartment from which TLR9 is thought to signal[39,173]. Hence, RAGE and DNA appear to remain associated during uptake and progression through the endosomal network, and the trafficking kinetics is influenced by the DNA structure and, potentially, the ability of these ligands to multimerize RAGE.

RAGE increases TLR9-dependent responses to suboptimal DNA stimuli

Both RAGE and TLR9 have been shown to activate the NF-κB family of transcription factors upon ligation of stimulatory ligands[34,133]. To assess the ability of CpG DNA to activate RAGE signaling, we employed an HEK cell line expressing a luciferase reporter gene under the control an NF-κB-inducible promoter. No significant reporter activation was observed upon DNA stimulation when these cells overexpressed RAGE alone (data not shown). A stable cell line expressing TLR9 together with TLR2 and tetracyline-inducible RAGE was established to evaluate the ability of RAGE to contribute to TLR9-dependent CpG-B responses. Two different length CpG-B oligonucleotides, which are optimal for stimulation of NF-κB via TLR9, activated NF-κB in a dose-responsive manner in cells expressing TLR9/2 without RAGE, and this activation was notably enhanced when RAGE expression was induced (**Figure 2.8a**, left panel). This RAGE-dependent enhancement was specific for DNA, as co-expression of RAGE did not alter NF-κB activation in response to the TLR2 ligand Pam3CysK4 (**Figure 2.8a**, right panel). We next sought to understand whether RAGE could similarly affect responses to mammalian DNA. For this, the luciferase reporter cell line was co-cultured with immortalized murine bone marrow-derived macrophages and treated with silica to induce macrophage cell death. Silica treatment of luciferase reporter cells alone did not induce significant NF-κB activation (data not shown). The presence of silica-killed macrophages in co-culture activated NF-κB and this activation increased in cells expressing RAGE

Figure 2.8 RAGE increases TLR9-dependent NF-κB activation in response to DNA ligands. (**a**) HEK293 cells expressing TLR9, TLR2, tetracycline-inducible RAGE and firefly luciferase under the control of the NF-κB-inducible ELAM promoter were treated with doxycycline to induce RAGE expression and then stimulated with CpG-B or Pam3CysK4 at the indicated concentrations for 12 hours prior to measurement of luciferase protein. (**b**) HEK293 cells expressing TLR9 and tetracycline-inducible RAGE-mCerulean ELAM-luc reporter were cultured with or without doxycyline to induce RAGE expression, together with murine bone marrow-derived macrophages. Co-cultures were left unstimulated or stimulated with silica crystals to induce macrophage cell death. Degradation of the phosphodiester (PO) CpG-B DNA stimulus served as a positive control for benzonase activity. Error bars indicate standard deviation. (**c**) Co-cultured cells from (b) were incubated with silica for 6 hours, stained with propidium iodide to label DNA from dead cells, and imaged by confocal microscopy. Silica was visualized using a reflection microscopy technique[1].

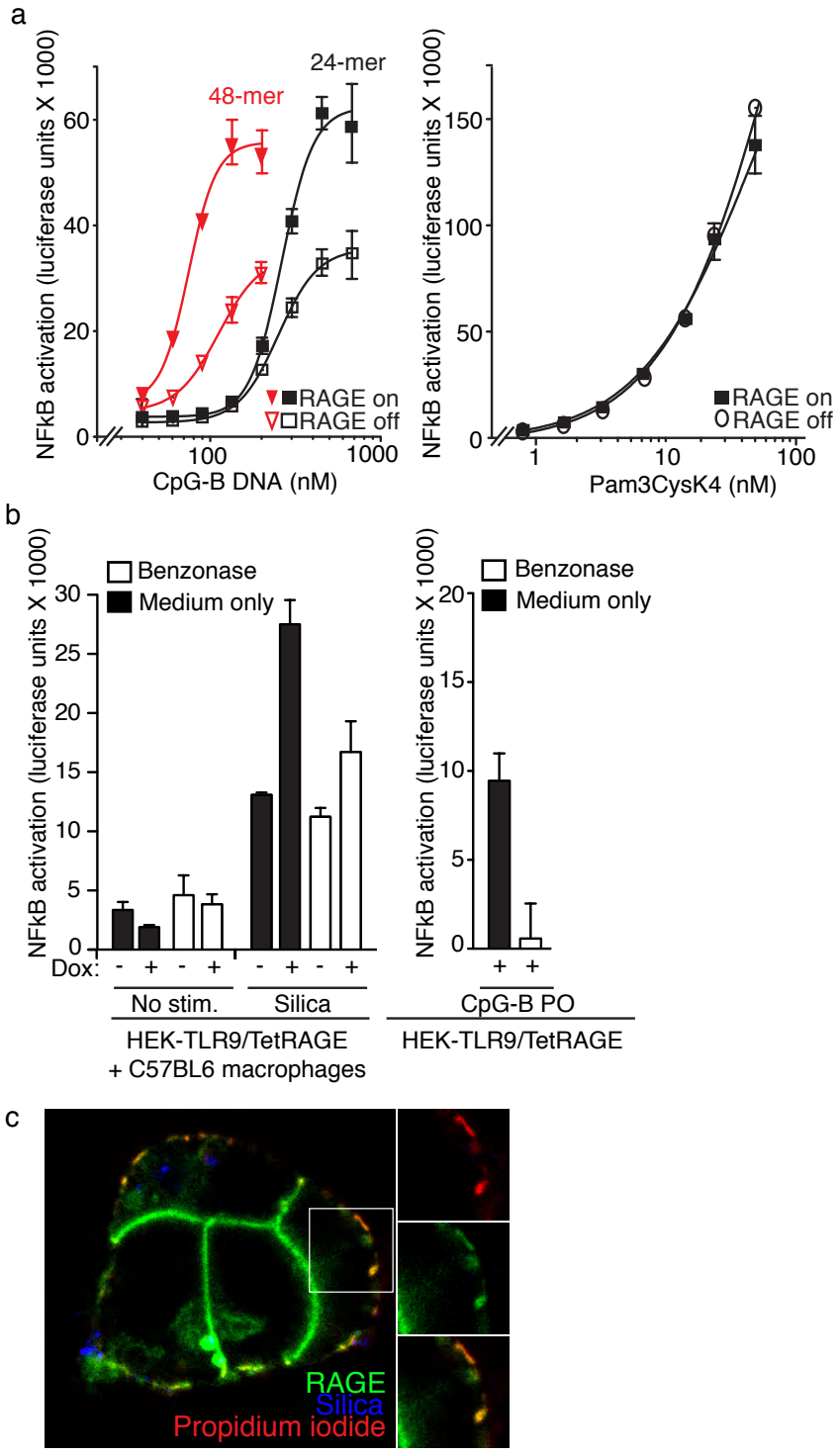


Figure 2.8 RAGE increases TLR9-dependent NF-κB activation in response to DNA ligands.

(**Figure 2.8b**). Treatment of co-cultures with benzonase to degrade extracellular DNA and RNA decreased the ability of dead macrophages to stimulate reporter cells, suggesting that enhancement of activation by RAGE is due largely to recognition of nucleic acids (**Figure 2.8b**). In effect, DNA from dying macrophages was seen interacting with RAGE when cocultured cells were imaged by confocal microscopy (**Figure 2.8c**). Taken together, these data suggest that RAGE effectively delivers DNA into the cellular compartments in which TLR9 is activated and that expression of RAGE sensitizes cells to both synthetic and genomic nucleic acids in the extracellular environment.

RAGE-deficient mice have impaired inflammatory responses to DNA in the lung

Given its ability to potentiate NF-κB activation in cultured cells, we sought to assess the role of RAGE in proinflammatory responses to DNA *in vivo*. RAGE is highly expressed in mammalian lung epithelia[140]; we therefore hypothesized that RAGE might participate in immune recognition of DNA in the airways. To address this possibility, we intranasally administered a low dose of CpG-B DNA to C57BL/6 wild-type or RAGE deficient (*ager*-/-) mice and evaluated inflammatory parameters in the lung at 24 hours after DNA exposure. Wild type mice mounted a strong inflammatory response to CpG-B DNA, characterized by a significant increase in neutrophils and the neutrophil granule product, myeloperoxidase, in bronchoalveolar lavage (BAL) fluid, and increased

expression of the neutrophil chemoattractant LIX (**Figure 2.9a**). Strikingly, RAGE-deficient animals showed significantly lower neutrophil influx, with MPO and LIX levels indistinguishable from PBS-treated control animals (**Figure 2.9a**). Histological analysis of lung tissue revealed marked perivascular and peribronchiolar leukocyte influx in wild type mice (**Figure 2.9b**, left panel) that was notably reduced in RAGE-deficient mice (**Figure 2.9b**, right panel). Analysis of cytokines in the BAL fluid revealed significantly reduced production of IFN-γ, IL-6, TNFα, and IL-12p70 in RAGE-deficient animals as compared to the wild type (**Figure 2.9c**). Of note, IL-1β and IFN-α, cytokines whose production is not directly activated by NF-κB, were also seen to be upregulated in wild type but not RAGE-deficient mice following CpG-B exposure, suggesting a broader role of RAGE in systemic inflammation. Taken together, these data indicate that RAGE expression plays an important role in the formation of an inflammatory response to immunostimulatory DNA in vivo, and particularly, in the lung.

Figure 2.9a-b RAGE mediates DNA-induced pulmonary inflammation *in vivo*. C57BL/6 wild-type or RAGE-deficient mice were administered PBS with or without 5 µg CpG-B via the intranasal route and analyzed 24 hours later for signs of pulmonary inflammation. n=6 mice per group. (**a**) Neutrophil cell number (left panel), myeloperoxidase concentration (MPO, middle panel) and LIX concentration (right panel) were determined in bronchoalveolar lavage fluid. Data shown are representative of two separate experiments (n=6 per group, per experiment) with similar results. Error bars indicate standard error of the mean and p values assessed by the nonparametric Mann Whitney U test are noted. ns = differences are not significant. (**b**) Representative hematoxylin and eosin stained paraffin sections of lung tissue from wild-type and RAGE-deficient (*ager* -/-) mice 24 hours after administration of 5 ug CpG-B. Black arrowheads indicate areas of apparent leukocyte infiltration in the wild type tissue and comparable areas in the *ager* -/- tissue.

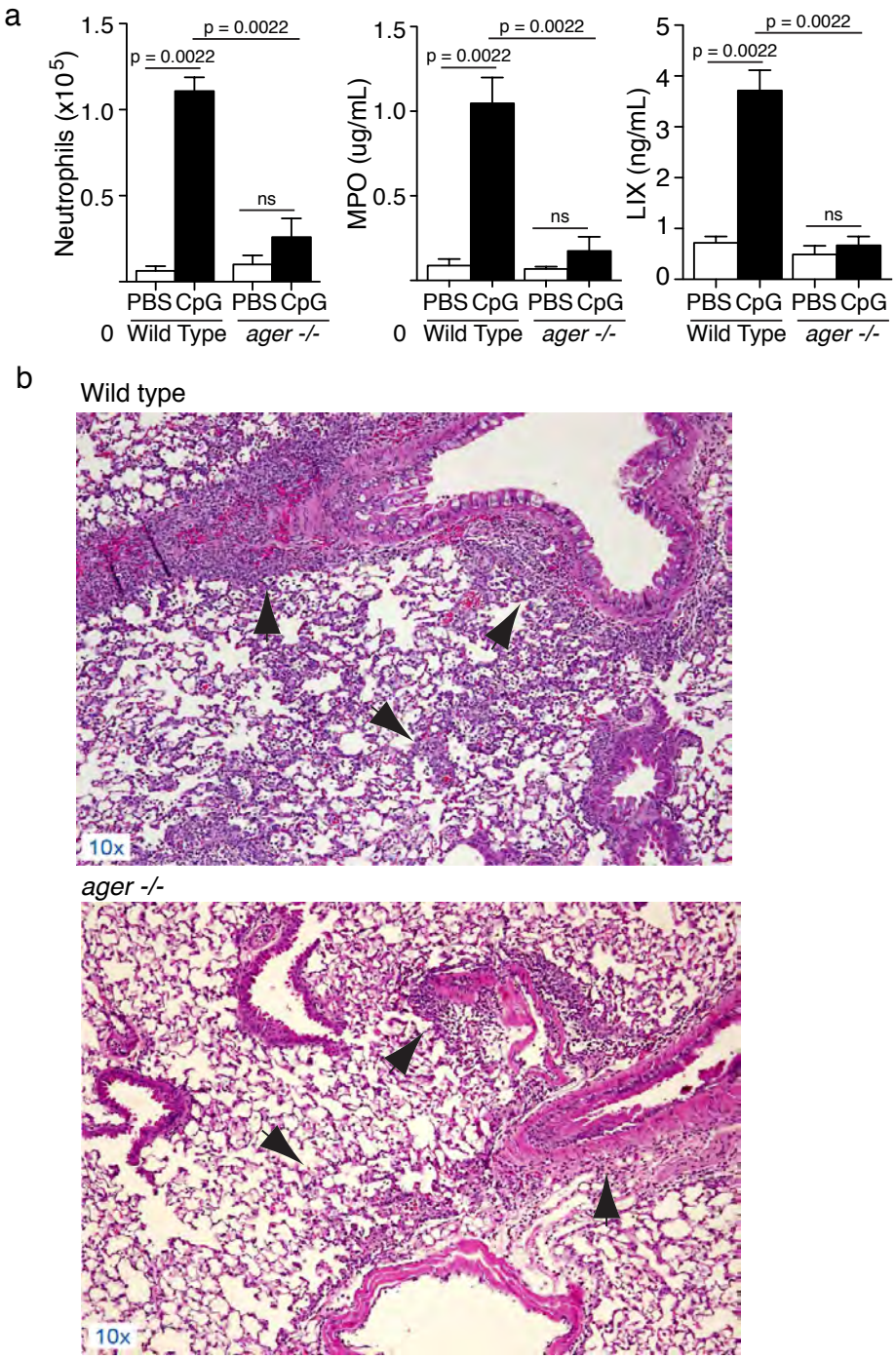


Figure 2.9a-b RAGE mediates DNA-induced pulmonary inflammation *in vivo*.

c

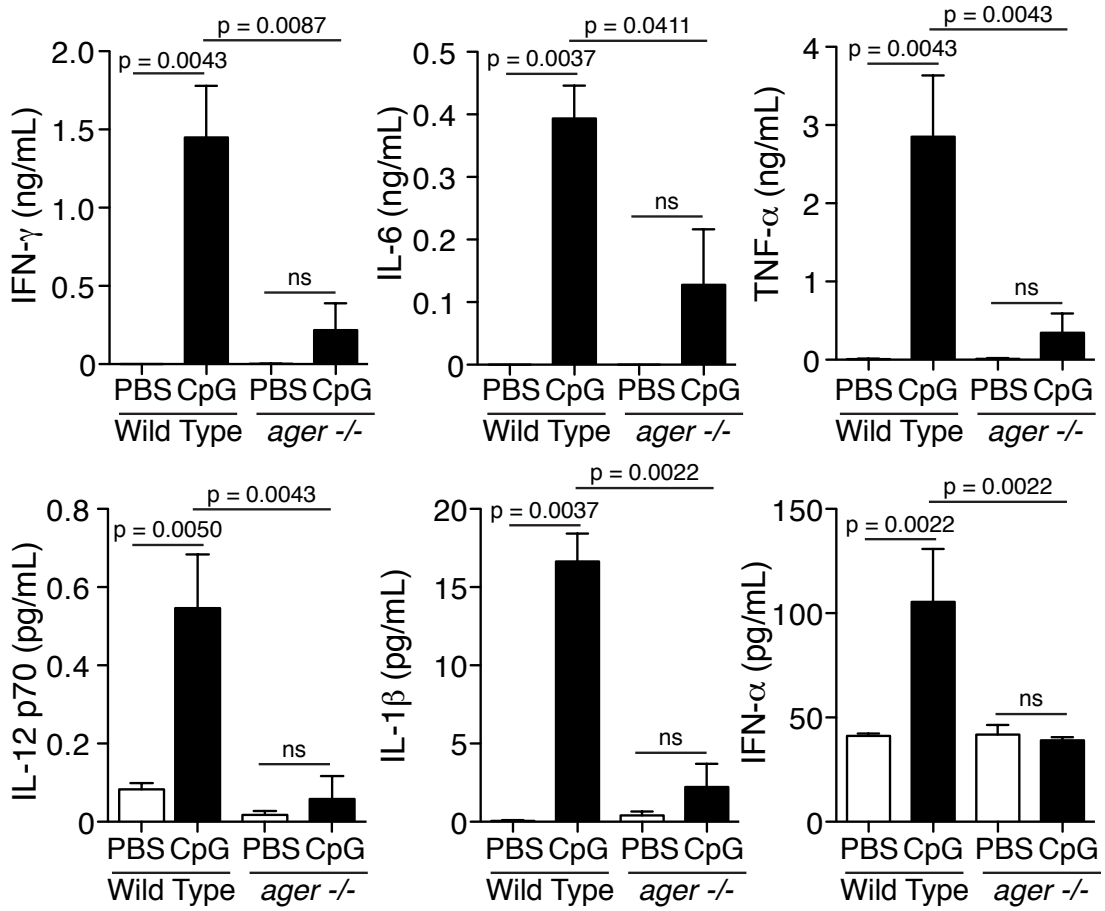


Figure 2.9c RAGE mediates DNA-induced pulmonary inflammation *in vivo*. C57BL/6 wild-type or RAGE-deficient mice were administered PBS with or without 5 µg CpG-B via the intranasal route and analyzed 24 hours later for signs of pulmonary inflammation. n=6 mice per group. (**c**) Cytokine concentrations in bronchoalveolar lavage fluid were determined by ELISA. Data shown are representative of two separate experiments (n=6 per group, per experiment) with similar results. Error bars indicate standard error of the mean and p values assessed by the nonparametric Mann Whitney U test are noted. ns = differences are not significant.

Discussion

The mechanisms of nucleic acid uptake from the extracellular milieu have long been a point of interest. Because nucleic acid-recognizing TLRs are expressed in endosomal compartments, early work on these receptors assumed the likelihood of a cell surface "uptake" receptor[39]. Discovery of such a receptor has been elusive, however, and the mechanism by which extracellular DNA gains access to intracellular receptors remains largely unsolved. A role in ligand uptake has been described for the TLR co-receptor protein CD14[101], integrins[102] and scavenger receptors[103,104]. However, few studies have shown direct biochemical evidence of DNA-receptor binding and an apparent functional redundancy complicates assigning an essential role to any single receptor.

Our work indicates that surface-expressed RAGE protein binds directly to nucleic acids and promotes their uptake into endosomal compartments, presumably facilitating their access to endosomal TLRs 3, 7, 8 and 9. While RAGE does not appear to be essential for DNA to access TLR9 in cultured cells, its expression notably enhances the ability of TLR9-expressing cells to activate the transcription factor NF-κB. The effect of RAGE is more dramatic *in vivo*, as mice lacking RAGE show a clear deficiency in their ability to mount a typical inflammatory response to TLR9-stimulatory DNA. These findings shed new light on the role of RAGE in inflammation.

It has been suggested that direct signaling of RAGE upon ligation of glycated proteins, amyloid-beta fibrils, S100 proteins and HMGB1 serves to initiate and perpetuate inflammatory responses[126,136,137,141]. Our work demonstrates a clear role for RAGE in uptake and intracellular trafficking of nucleic acid ligands; however, the occurrence or importance of a direct signal downstream of receptor ligation is, as yet, unclear.

Whether by simply modulating DNA uptake or by generating a regulatory signal, RAGE appears to be important in influencing the concentration threshold at which activation of inflammatory responses is achieved. We gave mice a suboptimal dose of a TLR9 stimulatory ligand, which in the absence of RAGE, was not able to induce inflammation. However, expression of RAGE at normal physiological levels permitted the animals to mount a potent inflammatory response to low concentrations of DNA. We speculate that such “thresholding” may serve to sensitize RAGE-expressing cells to the presence of DNA while allowing other cells to remain unactivated. The multi-ligand nature of RAGE may also contribute to thresholding of responses. It has been proposed that complexes containing multiple RAGE ligands and/or complexes capable of simultaneously ligating RAGE and other receptors (such as DNA-HMGB1-containing immune complexes seen in systemic lupus erythematosus) may provide a symphony of signals that allow the immune system to distinguish between distinct types of danger[175]. Such synergy between multiple RAGE ligands may help to reconcile our observation of a direct RAGE:DNA interaction

with published data indicating that HMGB proteins, also RAGE ligands, are required for TLR activation by nucleic acids[176].

The roles of co-receptors, such as HMGB1, and nucleic acid sequence in RAGE-mediated DNA responses is still somewhat unresolved. While our previous work suggested that a DNA:HMGB1 complex was more effective at activating plasmacytoid dendritic cells than HMGB1 alone[106], the binding studies presented here, which were carried out in the absence of HMGB1, indicate that HMGB1 is not essential for the RAGE-DNA binding event. However, HMGB1 is commonly present in mammalian serum and induced during tissue damage; thus, possible synergy between DNA and HMGB1 in the initiation of inflammation remains to be elucidated. With or without the help of co-receptors, specificity for distinct nucleic acid sequences or structures is a hallmark of many known nucleic acid sensors. Under normal physiological conditions, an unmethylated CpG dinucleotide motif[9] is required for activation of TLR9. Such a CpG sequence has also been reported as necessary for DNA binding to RAGE[128]. Our data, however, strongly suggest that the DNA-RAGE interaction occurs irrespective of nucleotide sequence. Co-crystalization of RAGE with bound DNA indicates that the binding is mediated entirely by interactions of the negatively charged phosphates of the DNA backbone with charged and hydrophilic residues at the RAGE:ligand binding interface. Additional binding studies using non-CpG oligonucleotides and base-free sugar-phosphate backbones, which also bound RAGE with no notable loss of affinity, further support a sequence-independent

binding modality. This lack of specificity seems to extend to RNA ligands, as both ssRNA oligonucleotides and base-free RNA backbones interacted with RAGE in a manner similar to DNA.

Charge-based binding is emerging as a generalized mechanism for interaction of RAGE with its ligands. It has previously been demonstrated that glycated BSA, which is highly acidic, may engage in electrostatic interactions with RAGE in a manner similar to that of dsDNA shown here.[129] Additionally, NMR spectroscopic analysis of calcium-bound S100b interaction with RAGE suggested that their interface resides adjacent to the area we designate “site 1” of the dsDNA binding surface[111]. Truncation of the acidic C-terminal tail region of HMGB1 has also been shown to significantly diminish its association with RAGE[177], suggesting that electrostatic interactions are key for any direct HMGB1 binding. It thus appears that the large positively charged surface of the RAGE V-C1 domains, of which only a portion is involved in dsDNA binding, may be able to engage distinct negatively-charged ligands through similar electrostatic attractions. Interestingly, charge-based interaction has also been suggested as the mechanism of DNA binding to other cell surface receptors[178,179] and may help to explain the apparent functional redundancy of receptors that interact with negatively charged ligands in the extracellular space.

While the interaction mechanisms described here are ostensibly applicable to any environment in which DNA and RAGE are present, physiological expression patterns of RAGE suggest that it may be particularly

important for inflammatory responses raised in the lungs. The predominant site of RAGE expression in adult mammals is in lung tissue[150], where the receptor is highly expressed on alveolar epithelial cells[140,180]. Several types of immune cells have also been described to express RAGE[150-153,181,182], though whether such expression is constitutive or inducible is not well defined. Various conditions can expose such cells to free DNA and RNA in the lung. For example, acute infections such as pneumococcal pneumonia, which is characterized by bacterial autolysis and release of foreign DNA into the lung[183], present a likely scenario in which nucleic acids would be accessible to a cell-surface receptor. Chronic pathological conditions, such as the accumulation of DNA-containing bacterial biofilms in cystic fibrosis[160] and the deposition of nucleic-acid containing immune complexes in the interstitial space of lupus patients[184], also expose lung cells to free extracellular DNA and RNA. Moreover, inflammation in the lung is often characterized by recruitment of neutrophils and formation of neutrophil extracellular traps (NETS), which involves extrusion of the cell's own DNA[185]. These and other situations suggest that recognizing extracellular nucleic acids of foreign or host origin may serve a broad function in detecting infection and tissue damage in the lung. The surface receptor protein RAGE, through its ability to concentrate DNA and RNA on cells and promote their uptake into specific receptor-containing intracellular compartments, may play a key role in regulating the initiation of immune responses to nucleic acids.

Materials and Methods

Oligonucleotides

Oligonucleotide (ODN) sequences and sources are listed in **Table 2.2**. Base-free phosphodiester and phosphorothioate backbones were a kind gift from Dr. Herman Wagner (Technical University Munich, Germany).

Cell Lines

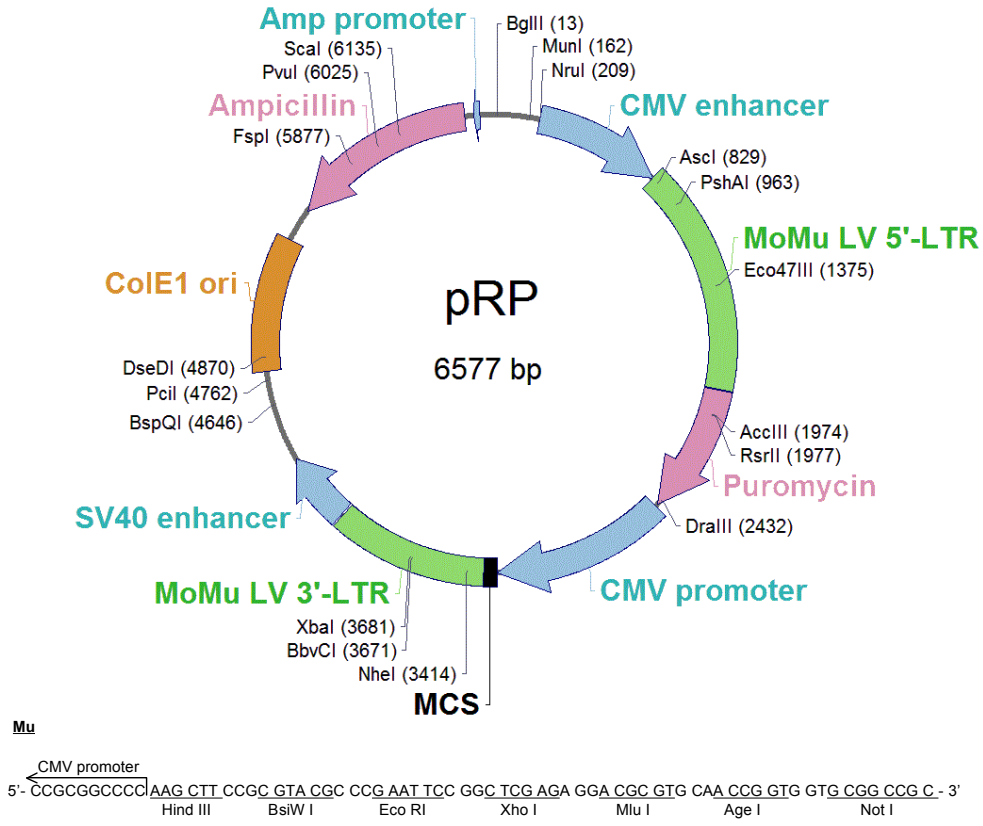
Cell lines in the HEK293, 293T and HeLa backgrounds were generated using retro- or lentiviral transduction with human RAGE C-terminally fused to fluorescent protein tags. cDNA encoding the human RAGE 404 amino acid transmembrane protein isoform (GenBank accession number AY755619) was amplified by PCR using upstream primer 5'-AAAACTCGAGATGGCAGCCGGAACAG-3' and downstream primer 5'-AAAAAGATCTAGGCCCTCCAGTACTACTCTCG-3'. RAGE cDNA and cDNA encoding CFP or YFP were sequentially subcloned into the murine moloney leukemia virus-based retroviral packaging vector pRP (**Figure 2.10**, produced by Brian Monks). To produce transducing retroviruses, 293T cells (220,000 per well in 96-well plates) were simultaneously transfected with pRP (100 ng) and plasmids encoding HIV gag-pol (100 ng) and VSV-G (10 ng) using TransIt reagent (Mirus Bio). Culture supernatants containing retrovirus were added to pre-existing target cell lines of interest and RAGE-positive cell lines were selected based on resistance to puromycin. Limiting dilution was used to

Table 2.2 Oligonucleotide sequences and sources.

Table 2.2 Oligonucleotide sequences and sources

	Sequence (5'-3')	Notes	Source(s)
ODN 752	CCATGACTGTAGGAAACTCTAG	Used in 2.8 Å crystal, sense strand	IDT
ODN 753	GCTAGAGTTTCCTACAGTCATG	Used in 2.8 Å crystal, antisense strand	IDT
ODN 981	CTGCAACGATGCTACGAACGTG	Used in 3.1 Å crystal, sense strand	IDT
ODN 982	CACGTTCGTAGCATCGTTGCAG	Used in 3.1 Å crystal, antisense strand	IDT
ODN 1826	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T	CpG-B, optimal mouse-stimulatory	TriLink
ODN 2006 PS	T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T	CpG-B, optimal human-stimulatory	IDT, Operon
ODN 2006 PO	TCGTCGTTTTGTCGTTTTGTCGTT	CpG-B, human stimulatory, phosphodiester	IDT, Operon
ODN 2336	G*G*GGACGACGTCGTGG*G*G*G*G*G	CpG-A	Coley, IDT, Operon
ODN 2395	T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G	CpG-C	Operon
ORN 9.2s PO	rArGrCrUrUrArArCrCrUrGrUrCrCrUrUrCrArArUrU	GFP siRNA-derived sequence, sense strand	IDT
ORN 9.2s PS	rA*rG*rC*rU*rU*rU*rA*rA*rC*rC*rU*rG*rU*rC*rC*rU*rU*rC*rA*rA*rU*rU	GFP siRNA-derived sequence, sense strand	IDT

Linkages are phosphodiester bonds between deoxynucleotides unless indicated: *,phosphorothioate linkage; r, ribonucleotide
Labeled ODN had biotin (TEG linkage) or AlexaFluor 647 (AmC7 linkage) attached to the 3' end.
Sources: Trilink BioTechnologies, Inc., San Diego, CA; Integrated DNA Technologies (IDT), Coralville, IA; Eurofins MWG Operon, Huntsville, AL.
Multiple lots of ODN were used throughout studies with no appreciable differences in activity.



CMV enhancer: bases 212-799
MoMu LV 5'-LTR: bases 800-1853
Puromycin resistance gene: bases 1860-2459
CMV promoter: bases 2470-3273
Multiple cloning site: bases 3276-3337
MoMu LV 3'UTR: bases 3341-4045
SV40 promoter: 4055-4383
SV40 origin: 4294-4379
Lac promoter: 4469-4498
ColE1 origin: bases 4807-5426
Ampicillin resistance gene: bases 5581-6441
Amp promoter: bases 6483-6511

Figure 2.10 pRP retroviral transduction vector.
RAGE-fluorescent protein chimeras were cloned into the vector at the BglII restriction site.

produce clonal cell lines.

For the tetracycline-inducible RAGE cell line, cDNA encoding human RAGE and the fluorescent protein mCitrine or mCerulean were cloned into the pEN_TTmcs entry vector (Addgene 25755) and transferred to the pSLIK-hygro vector (Addgene 25737) by recombination (both plasmids were the gift of Dr. Iain Fraser and are commercially available through Addgene). pSLIK plasmids were delivered using a lentiviral transduction system[186].

Binding Studies

Recombinant protein production is described in Additional Detailed Methods. In-vitro binding of biotinylated oligonucleotides to purified, his-tagged, human RAGE extracellular domain (V-C1-C2) was assayed using the AlphaScreen amplified luminescent proximity assay (Perkin Elmer)[186]. Titrations of oligonucleotides were incubated with 40 nM RAGE-his in buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% ultrapure BSA, 0.01% Tween 20) at 22 ºC for 30 minutes, followed by addition of streptavidin-conjugated donor beads and nickel-chelate acceptor beads for 10 minutes or longer. Fluorescence was analyzed with an Envision plate reader (Perkin Elmer). For confocal imaging studies of DNA binding to the cell surface, HeLa cells expressing RAGE-CFP and Rab4a-YFP or only Rab4a-YFP, were cultured on glass-bottom dishes (MatTek) overnight, then incubated with 1 µM ODN 2336-AlexaFluor 647 at 37 ºC for 5 minutes, washed with medium, and imaged by confocal microscopy. For flow cytometry analysis of

DNA binding to RAGE-positive and RAGE-negative cells, 293T cells expressing tetracycline inducible RAGE-mCitrine were cultured overnight in medium with or without 10 µg/mL tetracycline, then incubated with 1 µM Alexa 647-labeled ODN 2336 on ice for 30 minutes. Cells were washed with PBS and analyzed on a BD LSR II instrument (Becton Dickinson) using BD FACS Diva (Becton Dickinson) and FlowJo software (Treestar, Inc.).

DNA Uptake Studies

Uptake of DNA by RAGE-positive versus RAGE-negative cells was assessed using a tetracycline-inducible RAGE-mCitrine cell line. Cells in multiwell plates were cultured overnight in medium with or without 10 µg/mL tetracycline, then incubated with the indicated concentrations of AlexaFluor 647-labeled ODN 2006, 2336 or transferrin for 30 minutes on ice or at 37 ºC. Cells were washed with PBS and analyzed on a BD LSR II instrument (Becton Dickinson) using BD FACS Diva (Becton Dickinson) and FlowJo software (Treestar). For confocal microscopy analysis of DNA uptake and trafficking, HeLa cells stably expressing RAGE-CFP and either Rab5a-RFP or Rab9a-YFP were cultured on glass-bottom dishes (MatTek) overnight. Cells were “pulsed” with 500 nM Alexa 647-labeled ODN in medium for 5 minutes at 37 ºC, washed with PBS, and then incubated in medium for the indicated time periods prior to imaging. For quantification of RAGE and DNA colocalization with early and late endosomes, total labeled endosomes were manually counted in 3-6 cell images. Endosome spots

fluorescing in all three channels (endosomal marker, RAGE, ODN), were considered “triple-positive.”

Confocal Microscopy

Live cells were seeded at 20,000 - 30,000 cells in 100 µL medium on glass-bottom culture dishes (MatTek) and cultured overnight prior to treatment and imaging. Images shown represent a single z-plane though the approximate center of the cells of interest. Images were obtained with Leica SP2 AOBS or SP5 SMD confocal microscopes. Sequential scanning was used to avoid cross excitation between fluorescence channels.

Förster Resonance Energy Transfer (FRET) Measurements

HEK293 cells coexpressing RAGE-CFP and RAGE-YFP were brought into suspension and incubated in PBS with the indicated CpG ODN on ice for 20 minutes. After further incubation at 37 ºC for 5 minutes, the cells were washed with ice-cold PBS and kept on ice until flow cytometry FRET analysis was performed as previously described[187]. RAGE-CFP or RAGE-YFP single transfectants were used as controls. Microscopy-based sensitized emission FRET was performed using a Leica SP5 SMD confocal microscope. HEK293 cells were cultured on glass-bottom dishes and incubated with 10 µg/mL doxycycline for 24 h to express tetracycline-inducible RAGE-mCerulean and RAGE-mCitrine alone (as controls) or together. Cells were then treated with 0.25

μM AlexaFluor 647-labeled ODN 2006 or 2336 for 15-30 minutes at 37 ºC and imaged. FRET efficiency in areas of DNA:RAGE interaction was assessed by Leica TCS software, color coded as indicated, and representative cells were graphed.

RAGE Crosslinking

The amine-reactive crosslinking agent bis(sulfosuccinimidyl) suberate (Pierce) was prepared as a stock solution in water and diluted in PBS to working concentrations. Adherent HEK293 cells expressing human RAGE-YFP were detached from culture flasks and washed 3 times with PBS to remove amine-containing serum components. 300,000 cells per sample were incubated in PBS with or without crosslinker for 30 minutes on ice. Cells were then gently pelleted by centrifugation, BS3-containing supernatant was removed, cells were lysed in Laemmli buffer (63 mM Tris-HCl, 10% glycerol, 2% sodium dodecyl sulfate, 0.0025% bromophenol blue), and samples were analyzed by denaturing gel elctrophoresis and immunoblotting with antibody directed against the YFP tag (anti-GFP mAb, Clontech). Identical results were obtained in similar experiments with cells expressing tetracycline-inducible RAGE labeled with a monomeric Citrine tag, indicating that the weak dimerization potential of YFP was not the sole driver of chimeric RAGE dimerization in these cell lines.

Luciferase Reporter Assays

HEK293 cells stably expressing human TLRs 2 and 9, tetracycline-inducible human RAGE-mCerulean and a firefly luciferase gene under the control of the NF-κB inducible ELAM promoter were incubated overnight with 10 µg/mL doxycycline to induce RAGE expression, or left uninduced. Cells were stimulated as indicated for 8-16 hours and luciferase activity was assessed using SteadyGlo substrate (Promega) and an Envision multiwell plate reader (Perkin Elmer). HEK293 cells stably expressing human TLR9 and tetracycline-inducible human RAGE-mCerulean were co-cultured with immortalized mouse C57BL6 macrophages alone or with silica (Min-U-Sil 15, Western Reserve Chemical) for 6 hours prior to luciferase analysis or confocal microscopy.

Mice and *In Vivo* Studies

RAGE deficient (*ager-/-*) mice were produced by Taconic Artemis Pharmaceuticals (Cologne, Germany). Experiments were approved by the MedImmune, LLC internal Institutional Animal Care and Use Committee (IACUC), protocol MI-09-007. Mice were anesthetized with isofluorane prior to intranasal inoculation of 5 µg of CpG-B ODN 1826 in a total volume of 50 µl of PBS. 24 hours after DNA administration, bronchoalveolar lavage fluid (BALF) was collected by 3 x 0.6 ml washes with PBS/10mM EDTA/20mM HEPES, and lungs were harvested for histology. Lung tissue was inflated with 10% formalin prior to paraffin embedding, sectioning and staining with hematoxylin and eosin. Cytokines in BALF were

assessed by an MSD platform (MesoScale Diagnostics). IFNα (PBL Interferon Source), and LIX (R&D Systems) were measured by ELISA. Myeloperoxidase was quantified with a kit from Cell Sciences. Data shown are from one of two independent experiments with similar results.

Structural Determination

Human RAGE V-C1 domain (residues 23-237) was expressed in bacteria and purified using metal-ion affinity, hydrophobic interaction, and size-exclusion chromatography (see Additional Detailed Methods). V-C1:DNA complexes were crystallized and the structures determined by molecular replacement with ideal dsDNA and the V-C1 domain structure as search models [111,129]. Model building and refinement were carried out as detailed in Additional Detailed Methods and in **Table 2.1**. The structures and X-ray diffraction data were deposited at the RCSB protein data bank with accession numbers 3S58 and 3S59.

Statistical Analyses

Error bars in figures represent standard error of the mean for multiple samples, unless otherwise noted. Data sets were assessed for normality and significance of differences (p value) in cell culture-based assays was evaluated using unpaired t-tests, with alpha defined as 0.05. Significance of differences in animal studies was evaluated using nonparametric Mann-Whitney U tests, with alpha defined as 0.05. U Tests compared the untreated and DNA-treated group of the

same genotype, and the DNA-treated group of each genotype, as indicated. Prism software (GraphPad Software, Inc.) was used for all statistical analyses.

Additional Detailed Methods

Protein expression and purification

Protein for AlphaScreen binding studies: His-tagged human RAGE extracellular domain and deletion mutants containing the V domain (amino acids 1-121), V-C1 domains (amino acids 1-258) or C1-C2 domains (amino acids 122-342) were amplified by PCR and cloned into a his-tag containing plasmid. For the C1-C2 deletion mutant, the RAGE leader sequence was added to the constructs by overlapping extension PCR. To generate recombinant RAGE deletion mutant proteins, expression vectors were transfected into Freestyle 293F cells (Invitrogen) and his-tagged proteins were subsequently purified from the cell culture supernatants using a HisTrap column (GE Healthcare) and a DuoFlow FPLC system (BioRad). Proteins were eluted from the column in high salt buffer containing 500 mM sodium chloride and 25 mM imidazole, and dialyzed against PBS.

Protein for structural analysis: The V-C1 domain (Ala23 to Glu237) of human RAGE was cloned into a pET30a vector (EMD Biosciences) with a TEV cleavable N-terminal GB1 tag. Transformed BL21 (DE3) Codon Plus RIPL cells

(Stratagene) were grown at 37 °C until OD600 reached 1.2. Cells were then induced with 0.2 mM IPTG at 18 °C for 4 hours, harvested and resuspended in a buffer containing 100 mM NaCl, 5 mM imidazole and 20 mM Tris-HCl pH 8.0, supplemented with DNase- (Biomatik) and protease inhibitors (Roche Applied Science). Cells were lysed by sonication, and soluble protein was purified from cleared cell lysate by HisPrep IMAC column (GE Healthcare Bio-Sciences). Non-specific DNA contaminants were removed by adding ammonium sulfate powder to the IMAC elution fractions to a final concentration of 3 M. The protein pellet was dissolved in a buffer containing 100 mM NaCl and 20 mM Tris-HCl, pH 8.0, before TEV protease cleavage of the expression tag. The RAGE V-C1 domain was further purified by a second IMAC column, followed by phenyl-Sepharose hydrophobic interaction chromatography and size exclusion chromatography.

Crystallization

DNA oligos were synthesized by Integrated DNA Technologies without a 5'-phosphate. dsDNA with different sequences and lengths were tested in co-crystallization with the RAGE V-C1 domain. The two 22mer dsDNA oligos used in the current crystal structures were derived from vaccinia virus genomic repeat sequences (annealed from 5'-CCATGACTGTAGGAAACTCTAG-3' and 5'-GCTAGAGTTTCCTACAGTCATG-3') and CpG sequences (annealed from 5'-CTGCAACGATGCTACGAACGTG-3' and

5’-CACGTTCGTAGCATCGTTGCAG-3’). Oligos were dissolved in a buffer containing 100 mM KCl, 5 mM DTT, 20 mM HEPES, pH 7.4. Complementary oligos were mixed at a 1:1 molar ratio, heated to 95 °C, and annealed by slow cooling to room temperature. Annealed dsDNAs were added to diluted protein solutions (1 mg/ml) and the protein-DNA complexes were concentrated with centrifugal concentrators (Millipore) to 10-20 mg/ml before setting up crystallization using the hanging drop vapor diffusion method. The RAGE V-C1:DNA complex was crystallized with a well solution containing 12% PEG6000, and 0.1 M Tris-HCl, pH 7.4. A solution containing 12% PEG6000, 10% ethylene glycol, 10% glycol, and 0.1 M Tris-HCl, pH 7.4, was used as a cryoprotectant to freeze crystals in liquid nitrogen for data collection.

X-ray diffraction, structure determination and refinement

X-ray diffraction data were collected at GM/CA-CAT at the Advanced Photon Source (APS, Argonne, IL). Data were processed with the HKL200 program suite[188] and XDS[189]. The RAGE V-C1-DNA structures were determined by molecular replacement with Phaser[190] from the CCP4 program suite[191]. Previously reported RAGE structures[111,129], and ideal dsDNAs from Coot[192] or make_na server[193] were used as the initial search models. Structure building and refinement were carried out with Coot and Phenix[194]. The final refined models show no gross structural changes for RAGE upon DNA binding. DNA base pair hydrogen bonding restraints were generated by the PDB to 3D restraints

server[195] and employed during refinement. TLS parameters were generated by TLSMD server[196] and Phenix[194] and applied throughout the refinement, as well as twinned refinement protocols as suggested by the Xtriage program from Phenix[194]. Final structural models were validated by the Molprobity server[197] and the RCSB ADIT validation server[198]. Electrostatic charge surfaces were calculated with Delphi software[199] and displayed in Pymol (Delano Scientific LLC, San Carlos, CA).

PREFACE to Chapter III

Figure 3.2 in this chapter contains data excerpts from an article previously published in the journal *Nature Immunology*:

Tian J, Avalos AM, Mao S-Y, Chen B, Senthil K, Wu H, Parroche P, Drabic S, Golenbock D, **Sirois C**, Hua J, An LL, Audoly L, La Rosa G, Bierhaus A, Nawroth P, Marshak-Rothstein A, Crow MK, Fitzgerald KA, Latz E, Kiener PA, Coyle AJ. **Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE**. 2007. *Nature Immunology* 8 (5):487-496.

Data in Figure 3.2a were generated by E.L. using cells produced by C.M.S.

Data in Figure 3.2b-c were generated by J.T.

Chemical structure diagrams in Figure 3.3 were obtained from WikiMedia Commons (http://commons.wikimedia.org) and are not subject to copyright.

All other figure components in this chapter represent unpublished data generated by C.M.S.

CHAPTER III: Nucleic acid complexes are optimal RAGE ligands

Abstract

Complexation with specific antibodies and/or DNA- and RNA-binding proteins are common presentations of immunogenic nucleic acids *in vivo*. We sought to address the ability of such complexes to interact with the receptor for advanced glycation end products (RAGE) and the functional implications of these interactions. Here we show that DNA-containing immune complexes as well as DNA-HMGB1 complexes interact directly with RAGE. Complexation of HMGB1 with certain types of DNA modifies its binding to RAGE and its ability to activate production of type-I interferon by plasmacytoid dendritic cells. Additionally, nucleic acid-like posttranslational modification (poly-ADP ribosylation) of HMGB1 may similarly influence the ability of this ligand to interact with RAGE. These findings suggest that nucleic acid-containing multimolecular complexes may be a functionally significant format for RAGE ligands *in vivo*.

Introduction

Aside from experimental situations, extracellular nucleic acids are unlikely to persist in the body in “naked” forms. A plethora of chromatin-associated structural proteins, polymerases and transcription factors associate with DNA and RNA in the intracellular environment. Events that liberate self nucleic acids are likely to also release many of these associated proteins. The same can be said of microbial nucleic acids released by autolysis and other forms of bacterial death in tissues. Thus, uncomplexed nucleic acids are likely to be truly rare in physiological contexts of immunity. While reductionist studies of purified nucleic acid ligands have lent important insights into the essential nature of DNA or RNA for activation of innate immune receptor proteins, a complete understanding of immune responses to nucleic acid-containing ligands requires consideration of the forms these ligands acquire *in vivo*.

The importance of nucleic-acid containing complexes in activating the immune system has become clear in studies of certain autoimmune diseases, particularly systemic lupus erythematosus (SLE) and Sjögren’s syndrome. These diseases are characterized by the production of antibodies with specific paratopes for self components such as chromatin, RNA and associated proteins[200]. Once in the circulation, these so-called “autoantibodies” can bind to nucleic acid debris associated with normal cell turnover, forming antibody-ligand aggregates known as immune complexes. In the absence of autoantibodies,

such debris is effectively degraded and disposed of by nucleases, proteases and the body's scavenger functions. However, the formation of immune complexes appears to promote recognition of self nucleic acids and inflammation[201]. Many nucleic acid receptors preferentially activate signaling pathways that produce type-I interferons, and these cytokines play an important role in the pathogenesis of autoimmune diseases characterized by immune complexes[202].

While the importance of antibody-based immune complexes in autoimmune diseases is quite clear, less attention has been paid to the complexing of nucleic acids by non-antibody proteins. Recently, the mammalian antimicrobial peptide LL37 has been shown to associate with and complex extracellular DNA, and promote interferon production through activation of TLR9[203]. Complexation of endogenous nucleic acids by LL37 appears to be a key factor in breaking normal immune tolerance to self DNA and promoting the skin pathology of the autoimmune disease psoriasis. LL37 is found in psoriatic skin lesions, and has been shown to convert non-stimulatory "naked" genomic DNA into a stimulatory ligand for plasmacytoid dendritic cells, the key type-I IFN producing cell type[203]. Interestingly, LL37 associated with self DNA extruded from neutrophils in extracellular traps (NETs) has been shown to also play a key role in interferon production in SLE[204], suggesting that complexation of nucleic acids with this antimicrobial peptide can cause similar effects as antibody-nucleic acid complexes.

Somewhat more famous than LL37, another intracellular protein that has gained recognition as an extracellular effector molecule is HMGB1. This small 30 kDa protein was identified in studies of calf thymus chromatin proteins in the 1970s, as one of a “high mobilility group” (HMG) that moved rapidly in gel electrophoresis[205]. A subset of these proteins are characterized by one or more DNA-binding “box” domains, and are thus termed HMGB. HMGB1, the most studied of the mammalian HMG proteins, is a non-histone structural protein that binds to chromatin in the nucleus to facilitate DNA bending and transcription factor binding[206]. However, under conditions of cell stress or cell death, HMGB1 can be released into the extracellular environment, where it serves as a potent proinflammatory cytokine[207,208]. Many of the proinflammatory effects of HMGB1 have been traced to its interaction with RAGE on the cell surface[209]. Thus, HMGB1 is both a RAGE binding protein and a DNA binding protein, as well as an inflammatory mediator. This combination of attributes suggests that association of HMGB1 and DNA may affect RAGE in a synergistic manner.

Given the importance of nucleic acid complexes in the initiation of inflammation and the perpetuation of autoimmunity, we investigated the ability of RAGE to interact with nucleic-acid containing complexes.

Results

DNA-containing immune complexes interact with RAGE

Because naked nucleic acid ligands can effectively bind to RAGE in-vitro (see Chapter II), we sought to assess the relative binding of DNA-containing immune complexes to this receptor. Synthetic immune complexes can be generated by mixing immunogens with specific antibodies *in vitro*. We digested bacterial plasmid DNA with a multi-cutting restriction enzyme and incubated the fragments with a DNA-specific monoclonal antibody to generate dsDNA immune complexes. Cut DNA incubated with anti-dsDNA antibody showed retarded migration in agarose gel electrophoresis, consistent with immune complex formation (**Figure 3.1a**). Complexes generated in this manner were tested for their ability to bind directly to purified human RAGE extracellular domain in a physiological buffer solution. Complexes were titrated over a range to give DNA concentrations roughly equivalent to that of CpG-A and CpG-B oligonucleotide controls (*i.e.*, CpG oligos were used at a starting concentration of 200 nM, roughly equivalent to 2 µg, the amount of DNA used in each complex). Binding of immune complexes closely mimicked that of CpG-A DNA, with affinity in the low nanomolar range (**Figure 3.1b**). The CpG-A oligonucleotide is thought to aggregate into DNA-only complexes by virtue of G-tetrad formation between molecules. The fact that both this DNA-only complex and the DNA-antibody complex showed similar binding signal, while the unimolecular CpG-B bound less

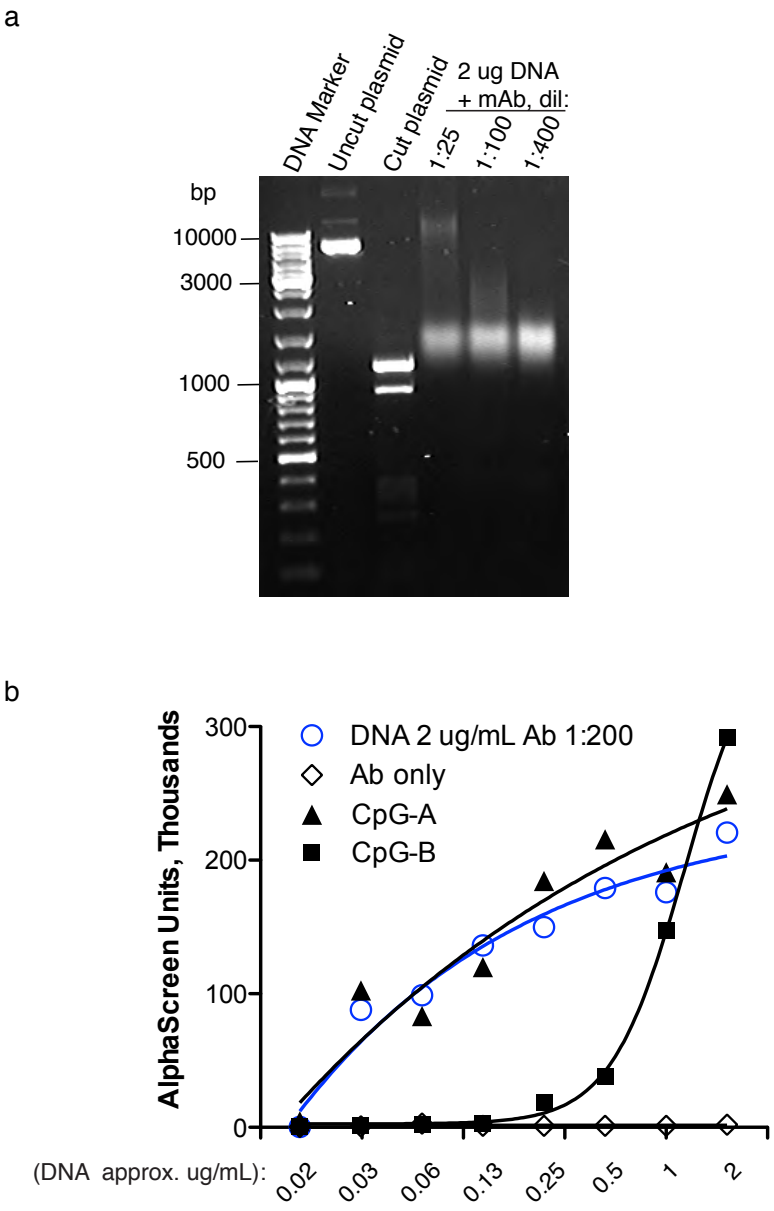


Figure 3.1 DNA:antibody complexes bind to RAGE.
(**a**) Characterization of DNA:antibody complexes by agarose gel electrophoresis. Electrophoretic mobility of complexes made from the indicated concentrations of cut plasmid DNA and anti-DNA monoclonal antibody were compared to plasmid DNA alone. Retardation of DNA complexed to antibody is seen as upward smearing of bands. (**b**) The ability of DNA:antibody complexes (open blue circles) to bind to 10 nM purified, his-tagged human RAGE extracellular region (V-C1-C2) was assessed by AlphaScreen binding assay. CpG oligonucleotides and antibody alone served, respectively, as positive and negative RAGE binding controls.

favorably, suggests that complexes interact preferentially with RAGE in this binding assay.

Complexes of HMGB1 and DNA are optimal RAGE ligands

Both HMGB1 and DNA have been reported to be direct RAGE ligands. However, because HMGB1 is a DNA binding protein, it is likely that these two ligands frequently exist as a complex in the extracellular environment. We first sought to visualize the relationship of an HMGB1:DNA complex with RAGE. In confocal microscopy studies, a complex of fluorescent CpG-A with HMGB1 was seen to associate with fluorescently-tagged RAGE on the cell surface (**Figure 3.2a**, left panel). As early as 10 minutes after addition, the complex and RAGE could be seen to colocalize in intracellular compartments resembling endosomes. By 60 minutes after addition, nearly all RAGE had been internalized from the cell surface and localized with HMGB1:DNA complexes within the cell (**Figure 3.2a**, middle panel). To confirm that these compartments were endosomes, we assessed the colocalization of fluorescently-tagged RAGE with fluorescently-labeled transferrin after a 60-minute incubation. Transferrin in complex with its specific receptor is known to enter early endosomes, where it releases its cargo of iron ions prior to transfer to recycling endosomes and return of the transferrin receptor complex to the cell surface[37]. Many RAGE-containing compartments were transferrin-positive after 60 minutes of incubation (**Figure 3.2a**, right panel) suggesting that RAGE-associated uptake of HMGB1:DNA complexes occurs via

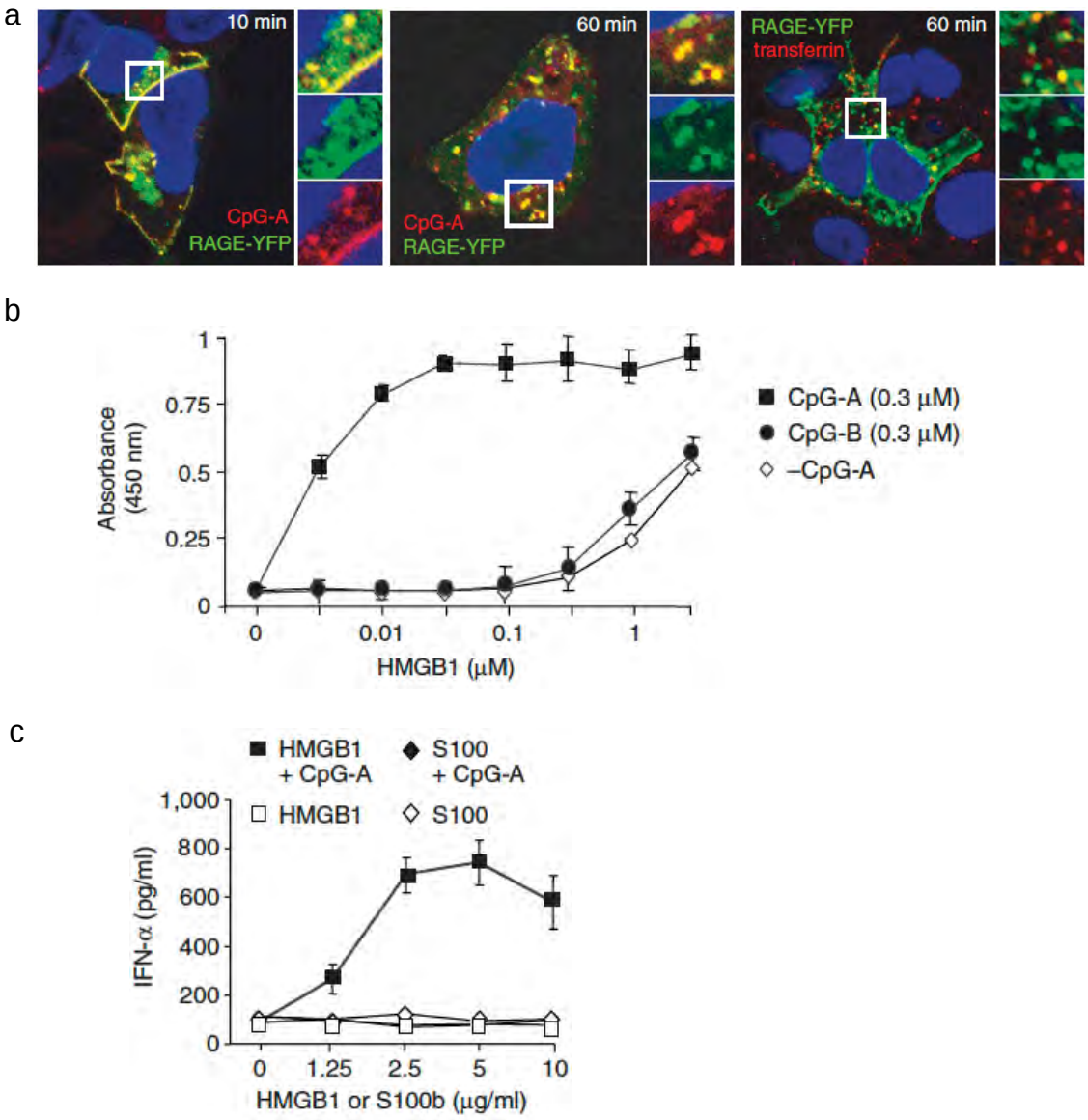


Figure 3.2 HMGB1:DNA complexes interact with RAGE and induce type-I interferon.

(**a**) HEK293 cells expressing RAGE protein tagged with YFP were stimulated with fluorescently-labeled CpG-A oligonucleotide or fluorescently-labeled transferrin for the indicated times and imaged by confocal microscopy. Cell nuclei are stained with Hoechst dye (blue). (**b**) Binding to immobilized RAGE of HMGB1 alone or complexed with CpG-A or CpG-B oligonucleotides was assessed in a plate-based binding assay. (**c**) Murine plasmacytoid dendritic cells were stimulated with HMGB1 or S100b alone, or with these ligands pre-incubated with CpG-A. Interferon-α in culture supernatants after 24 hours of stimulation was measured by ELISA.

an endosomal route, similar to that observed for DNA alone (see Chapter II).

Because both DNA alone and DNA complexed with HMGB1 seem to interact in a similar manner with RAGE on cells, we sought to identify differences in ligand:receptor interactions at the molecular level. We assessed the relative affinity of RAGE for HMGB1 alone versus DNA-complexed HMGB1 with a plate-based binding assay. A CpG-A:HMGB1 complex bound RAGE at a concentration more than 100 times lower than HMGB1 alone, or HMGB1 complexed to CpG-B oligonucleotide (**Figure 3.2b**). The large difference in signal between HMGB1 complexes containing aggregated CpG-A versus short, unimolecular CpG-B suggests that increased binding is not due to mere synergy between two RAGE ligands (HMGB1 and DNA), but rather to attainment of a large complex structure.

To evaluate this possibility on a functional level, we stimulated murine plasmacytoid dendritic cells (pDC) with HMGB1 or another RAGE ligand, S100b, either alone or combined with CpG-A. HMGB1 and S100b alone induced little interferon production by pDC in culture (**Figure 3.2c**). Complexation of HMGB1 with CpG-A, however, stimulated cells to produce IFN-α in a dose-dependent manner (**Figure 3.2c**). S100b is not thought to bind to DNA and is unlikely to form complexes. In keeping with this, a mixture of S100b and CpG-A was unable to induce strong IFN-a production from cultured pDC (**Figure 3.2c**). Taken together, these data suggest that increased binding to RAGE and downstream

cytokine production are preferentially induced not just by combinations of RAGE ligands, but by the multimolecular complex structures that they form.

Parylation of HMGB1 may contribute to RAGE binding

In addition to complexing with DNA, a post-translational modification, poly-ADP ribosylation ("parylation"), on HMGB1 may also enhance its interaction with RAGE. Poly ADP-ribose (PAR) is a polymer of ADP-ribose moieties derived from nicotine-adenine dinucleotide (NAD), and is covalently added to multiple sites on HMGB1 by poly ADP-ribose polymerases (PARP family members) in the cell nucleus[210,211]. Parylation of HMGB1 facilitates its dissociation from chromatin and subsequent passive diffusion out of the nucleus[211]. Once in the cytosol, HMGB1 can be released from the cell upon cell death or, in the case of monocytes and macrophages, through an active secretion process[212-215]. NAD monomers have a chemical structure similar to that of an RNA adenine dinucleotide (**Figure 3.3a**), and thus, PAR is structurally similar to polyadenosine RNA. Because RNA binds directly to RAGE (see Chapter II), we hypothesized that PAR might also be a RAGE ligand. We tested the ability of RAGE to bind to isolated PAR polymer using several variations of a plate-based binding assay. PAR and the RAGE extracellular domain showed detectable binding to each other, regardless of which of the two binding partners was immobilized on the plate (**Figure 3.3b**). Activated PARP parylates itself as well as other proteins; RAGE also bound to immobilized parylated PARP (**Figure 3.3b**). Taken together,

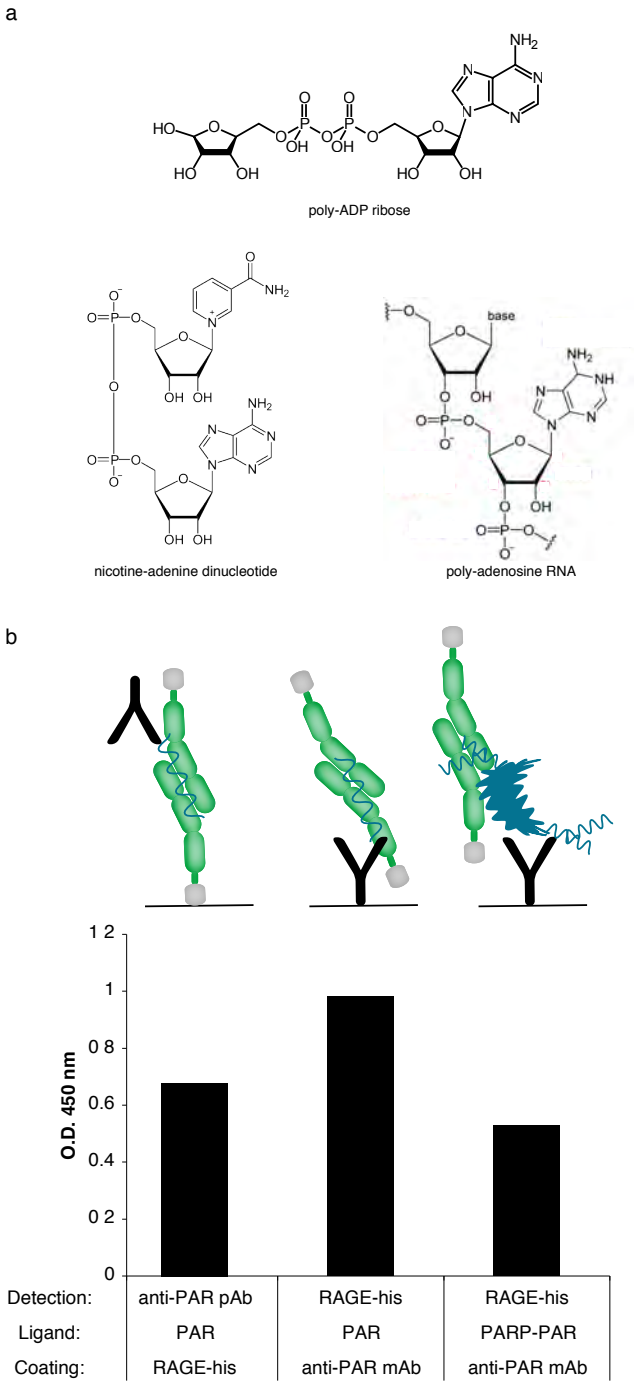


Figure 3.3 PAR is a RAGE ligand.
(**a**) Chemical structures of poly-ADP ribose (PAR), nicotine adenine dinucleotide (NAD) and an adenosine RNA dinucleotide. (**b**) Cartoon representations of plate-based binding assays (top) and signal representing relative RAGE:PAR binding (bottom). Background absorbance readings of negative controls = ~0.1.

these data suggest that RAGE is able to interact with parylated protein by virtue of direct binding to PAR adducts. Hence, parylation of HMGB1 might serve to increase its interaction with RAGE in a manner similar to complexation with DNA.

Discussion

The results presented here provide initial evidence that multimolecular complexes containing nucleic acids or similar molecules such as PAR are optimal RAGE ligands. Important future directions for this work include directly evaluating the ability of parylated HMGB1 to interact with RAGE, and the outcome of this interaction *in vivo*. Because glycohydrolases that remove PAR moieties exist in the cytosol[216], it is not yet clear whether HMGB1 is de-parylated before its release from cells, or if parylation could play a role in inflammatory activation by extracellular HMGB1. Parylated or not, there is strong evidence that HMGB1 is a component of naturally-occurring immune complexes, including those circulating in the serum of SLE patients[217,218]. Hence, the effects seen here for DNA-antibody complexes and DNA-HMGB1 complexes are likely to be similar, and perhaps further enhanced, when these components come together *in vivo*.

RAGE is expressed on many cell types, and exposure to particular ligands may vary depending on the tissue in which RAGE is expressed. Binding of individual ligands to RAGE has been shown primarily to initiate signaling pathways that result in activation of NF-κB. While NF-κB-induced cytokines certainly play a role in autoimmunity[219], type-I interferon has emerged as a key “signature” of nucleic acid recognition in several autoimmune syndromes[220]. As shown here, single RAGE ligands like HMGB1 and S100 alone may not be

sufficient to induce interferon production, but complexation of HMGB1 can attain this activation threshold. Type-I IFN production by pDC, presumably through TLR9, is preferentially activated by nucleic acids like CpG-A that adopt complex structures[221]. The data in this chapter provide preliminary evidence that multimolecular complexes of RAGE ligands may also activate this pathway, and suggest that receptor "clustering" may be a key event for IFN activation. Given the large amount of evidence that RAGE preferentially activates NF-κB upon receptor ligation (see Chapter I), type-I IFN activation may be a smaller role that RAGE plays specifically after binding nucleic acid-containing ligands. Nevertheless, because type-I IFNs enhance their own production via feedback loops, small increases in IFN activation may translate into large differences in autoimmune pathology and thus, RAGE:ligand interactions may be a contributing factor in the cytokine balance of autoimmune diseases.

Materials and Methods

Studies of DNA immune complexes

To generate immune complexes, plasmid pcDNA3 (Invitrogen) was digested with the restriction enzyme DpnI (Fermentas) to generate double-stranded DNA fragments. The indicated dilutions of monoclonal anti-dsDNA (MAB030, Chemicon) were mixed with 2 µg of cut DNA in PBS and incubated for approximately 1 hour at 4 ºC. Preparations were used immediately or frozen at -20 ºC until use. Samples (containing 2 µg of DNA per well) were electrophoresed on a 1% agarose gel containing trace amounts of ethidium bromide, in Tris-borate-EDTA electrophoresis buffer, and the gel was imaged using a Gel Dock XR System with Quantity One software (BioRad).

For the AlphaScreen binding assay, the indicated dilutions of immune complexes were incubated with 10 nM his-tagged human RAGE extracellular domain (V-C1-C2) and 1 µg/mL biotinylated anti-mouse IgG monoclonal antibody in assay buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% ultrapure BSA, 0.01% Tween 20) for 30 minutes at room temperature. Biotinylated CpG-A (ODN 2216, Integrated DNA Technologies) and CpG-B (ODN 2006 with a phosphodiester backbone, Integrated DNA Technologies) at the indicated concentrations were incubated with 10 nM RAGE-his in the same way. Streptavidin-conjugated AlphaScreen donor beads and nickel chelate acceptor beads (Perkin Elmer) were added to the reaction and incubated for an additional 20 minutes at room

temperature. Induced chemiluminescent signal was measured with an EnVision plate reader and associated software (Perkin Elmer).

Studies of HMGB1:DNA complexes

Confocal microscopy: HEK293 cells were retrovirally transduced with an expression vector encoding full-length human RAGE with a YFP tag fused to the C terminus (cytoplasmic domain). HEK cells stably expressing RAGE-YFP were seeded onto glass bottom tissue culture dishes (MatTek) and cultured for 16 hours in growth medium (DMEM, 10% FBS, 10 µg/ml ciprofloxacin). Cells were treated with Alexa647-conjugated human transferrin (Invitrogen) or a complex of HMGB1 (calf thymus derived, 3 g/ml; gift from MedImmune) and Bodipy 630/650 conjugated CpG-A (ODN 2216, 1 µM; Eurofins MWG Operon) for the indicated time periods. Nuclei were stained with Hoechst 33342 dye 5 minutes before the cells were imaged. After three washes in prewarmed growth medium, the cells were analyzed with a Leica TCS SP2 AOBS laser scanning confocal microscope. Sequential scanning was performed to ascertain separation of fluorescence emission with only one laser and the respective photomultiplier tubes active per scan.

Binding assay: Recombinant Fc-tagged human RAGE extracellular domain was coated onto 96-well plates. HMGB1 alone or complexed with CpG-A (ODN 2216, Invivogen) or CpG-B (ODN 2006, Invivogen) was added and binding was detected by biotin-labeled monoclonal antibody to HMGB1.

In vitro pDC stimulation: pDC were isolated from murine bone marrow cells (> 60% pure) using a plasmacytoid dendritic cell isolation kit (Miltenyi Biotec) and plated in 96-well plates at a density of 2.5 x 10^4 cells per well. Cells were stimulated for 24 hours with the indicated concentrations of ligands (HMGB1, produced internally at MedImmune; S100b, Calbiochem) alone, or pre-complexed with CpG-A (ODN 2216, Invivogen). IFN-α in cell culture supernatant was measured by ELISA (R&D Systems).

RAGE binding to PAR and parylated protein

Plate-based binding assay: 96-well high protein binding plates (Maxisorp, Nunc) were coated with PBS containing either 0.6 μM his-tagged RAGE extracellular domain (gift from MedImmune) or 2 μg/mL anti-PAR monoclonal antibody (Trevigen) at 4 ºC overnight. Wells were washed and then blocked with PBS containing 0.01% Tween 20 and 3% bovine serum albumin at room temperature for 1.5 hours. PAR polymer (10 ng/uL, Trevigen) or parylated PARP protein (200 nM) in blocking buffer were added for 2 hours at room temperature. After washing, bound PAR or parylated PARP as detected by addition of anti-PAR polyclonal antibody (1:4000 dilution, Trevigen) followed by HRP-conjugated anti-rabbit secondary antibody (BioRad) or 0.6 μM hRAGE-his followed by nickel-HRP (BioRad). Secondary antibodies alone and/or non-binding “ligands” plus secondary antibodies were used as negative controls.

Chapter IV: Discussion

The panorama of nucleic acid sensing

Proper sensing of the location and origin of nucleic acids is an essential process in maintaining structural homeostasis and in defending mammalian organisms from microbial and parasitic pathogens. Nucleic acid-sensing receptor proteins are situated in specific locales, *i.e.*, the extracellular face of cell membranes and the lumen of intracellular compartments, where the presence of DNA or RNA is indicative of cell damage or infection. The origin of nucleic acids in these locations, and even more importantly, in the cytosol, is further determined by their biochemical nature; for example, presence of unmethylated CpG dinucleotide motifs in DNA indicate bacterial or viral origin, while presence of a 5‘ triphosphate on RNA indicates that it was produced by a virus, not the mammalian cell. The combination of “location” and “origin” information provides a code that allows the body to distinguish a true threat to its integrity, requiring immune and repair responses, from a minor disturbance that requires only “clean up” of misplaced nucleic acids. Hence, proper interpretation of the location-origin code is vital for efficiently controlled responses to nucleic acids. Perhaps for this reason, an ever-increasing team of proteins intervene in various aspects of nucleic acid sensing and discrimination.

This “team” can be divided into three conceptual divisions: access control, facilitation, and response. “Access control” includes nucleases that simply eliminate DNA and RNA that they encounter, to prevent access of these

molecules to the “response” division. “Facilitator” proteins are receptors like RAGE, and nucleic acid-complexing agents like HMGB1 and LL37 that help to protect nucleic acids from nucleases and/or promote their uptake. Combinations of access control and facilitation influence the relative ability of nucleic acids to interact with the “response” division, true sensor proteins that check for “passwords” in the biochemical structure of the nucleic acid and activate signals.

RAGE, nucleic acids, and inflammasomes

The preceding chapters have considered the manner in which a facilitator protein, RAGE, interacts with nucleic acids in the extracellular environment and influences the activation of endosomal TLRs (response mediators). Despite this empirical focus, the data presented here also shed light on potential roles for RAGE in activation of immune sensors other than TLRs. Both Chapters II and III highlight ways in which “bulky” ligands, either multimers of CpG-A or complexes of DNA and proteins, interact preferentially with RAGE. Another bulky RAGE ligand, amyloid-β fibrils, has been shown to activate the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome, an IL-1β processing complex[222]. The essential activating stimulus for NLRP3 is still somewhat cryptic and several hypotheses have been put forward[223]. Fibrillar amyloid-β seems to induce inflammasome activation by disrupting the integrity of endolysosomes[222],

and the same mechanism of action has been shown for other activators, such as large crystalline structures[224]. There is, then, reason to speculate that large complexes taken up by RAGE into the endolysosomal network may also be able to "burst" these compartments in a manner similar to that shown for amyloid-β, leading to inflammasome activation. This might account for the finding that CpG DNA administered into the murine lung could induce IL-1β secretion into the airways in a RAGE-dependent manner (see Chapter II). While the CpG-B oligonucleotide used in those experiments does not form bulky structures on its own, complexation of the oligo with proteins *in vivo* (refer to Chapter III) might serve to create an inflammasome-activating stimulus. The endolysosomal rupture hypothesis is closely associated with another proposed mechanism of NLRP3 activation by reactive oxygen species (ROS)[223,225]. Although this thesis work did not find evidence of direct signaling of RAGE upon nucleic acid ligation, interaction of RAGE with its other ligands has been reported to induce ROS[139,141,142,226]. Hence, there are two potential mechanisms by which bulky and/or nucleic-acid containing RAGE ligand complexes may activate IL-1 via the NLRP3 inflammasome. It is furthermore interesting to note that both RAGE[227] and NLRP3[228] have been implicated in the pathology of atherosclerosis. RAGE activity has been proposed to explain the link between diabetes (in which AGEs are increased in the bloodstream) and vascular disease (which is exacerbated by inflammation)[229,230]. The ability of nucleic-acid containing RAGE ligands to exert effects similar to those of AGEs may thus contribute to the documented

connection between atherosclerosis and the immune complex disorder SLE[231-234].

Another IL-1-activating inflammasome complex is formed by the cytosolic DNA sensor absent in melanoma 2 (AIM2)[87,235]. AIM2 has been reported to interact with several kinds of double-stranded synthetic and pathogen-derived DNA[87,236-238]. While the evidence presented here shows that RAGE facilitates uptake of DNA into endosomes (see Chapter II) not cytosol, the endolysosomal rupture model suggests a mechanism by which nucleic acid RAGE ligands in intracellular compartments could also gain access to cytosolic AIM2. Hence, disruption of endolysosome integrity may be capable of inducing an IL-1 response via two distinct inflammasome receptor complexes. As IL-1β is one of the most potent inflammatory cytokines and an important mediator of both beneficial and pathological immune responses, this potential role for RAGE and nucleic acids in inflammasome activation warrants further experimental interrogation.

DNA, PARP, HMGB1 and RAGE: regulatory integration?

To again recall John Muir's words (see Chapter I), in attempting to "pick out" the ways in which HMGB1 might interact with RAGE, we found that, though perhaps not connected to "everything in the universe," it was certainly connected to many

processes in the nucleus, cytosol and extracellular environment. As discussed in Chapter III, HMGB1 both binds DNA and becomes poly-ADP ribosylated in the nucleus. Upon recognizing that poly-ADP ribose (PAR) is structurally similar to RNA, we became interested in its ability to interact with RAGE and found that it could do so (see Chapter III). This opens the possibility that HMGB1 may ligate RAGE via PAR and/or bound DNA. It is worth recalling that HMGB1 consists of two DNA-binding box domains (A-box and B-box), in addition to a 30 amino acid "tail" domain consisting solely of aspartic acid and glutamic acid residues. Direct HMGB1 binding to RAGE has been suggested to be mediated by this acidic tail[177], and the tail also appears to fold back on the box regions to block DNA binding sites[239]. It would therefore appear that acidic, negatively-charged molecules interact both with the box regions of HMGB1 and with the ligand binding region of RAGE (see Chapter II). It is thus conceivable that bound DNA could serve as a "bridge" between HMGB1 and RAGE, mediating indirect binding. Under other circumstances, parylation on HMGB1 might play a similar role in mediating indirect binding, while a direct interaction between the HMGB1 tail and RAGE could also occur. This variety of likely binding modalities suggests that the nature of the RAGE:HMGB1 interaction could vary depending on the manner in which HMGB1 is released from cells (parylated or not), the absence vs. presence of extracellular DNA, and the relative concentration of these factors in the environment. By this logic, direct ligation of RAGE by individual ligands (HMGB1 alone, nucleic acid alone, PAR alone) may promote different receptor

function than ligation of RAGE by a multimolecular complex of interacting ligands (refer to Chapter III, Discussion).

Beyond basic ligand-receptor considerations, cell stress responses relating to HMGB1 and parylation may also be tied to RAGE (**Figure 4.1**). As noted above, RAGE signaling appears to induce the generation of reactive oxygen species (ROS). ROS production during inflammation and cell stress is linked to DNA damage[240], and damaged chromatin is the activating trigger for poly-ADP ribose polymerases (PARP)[241]. PARP activation leads to HMGB1 parylation and release from the cell[211], which can then feed back on RAGE, to promote further inflammatory signaling. Additionally, extracellular signal-regulated kinases (ERK1/2), which are activated by RAGE, can activate PARP even in the absence of DNA damage[242]. Hence, RAGE signaling may upregulate the release of its own ligands (HMGB1 and other parylated proteins) from intracellular stores. Furthermore, PARP has been shown to directly co-activate transcription by NF-κB[243,244], the predominant transcription factor induced by RAGE (see Chapter I). Both the presence of PARP[243] and its acetylation by nuclear cofactors[244] were shown to be important for optimal NF-κB activation. This suggests that PARP regulation under cell stress conditions could modulate activation of NF-κB and its role in the RAGE feedback loop (refer to [134]). Taken together, the known connections between PARP, HMGB1, DNA, RAGE, kinases, and transcription factors suggest a complex regulatory network in which all of

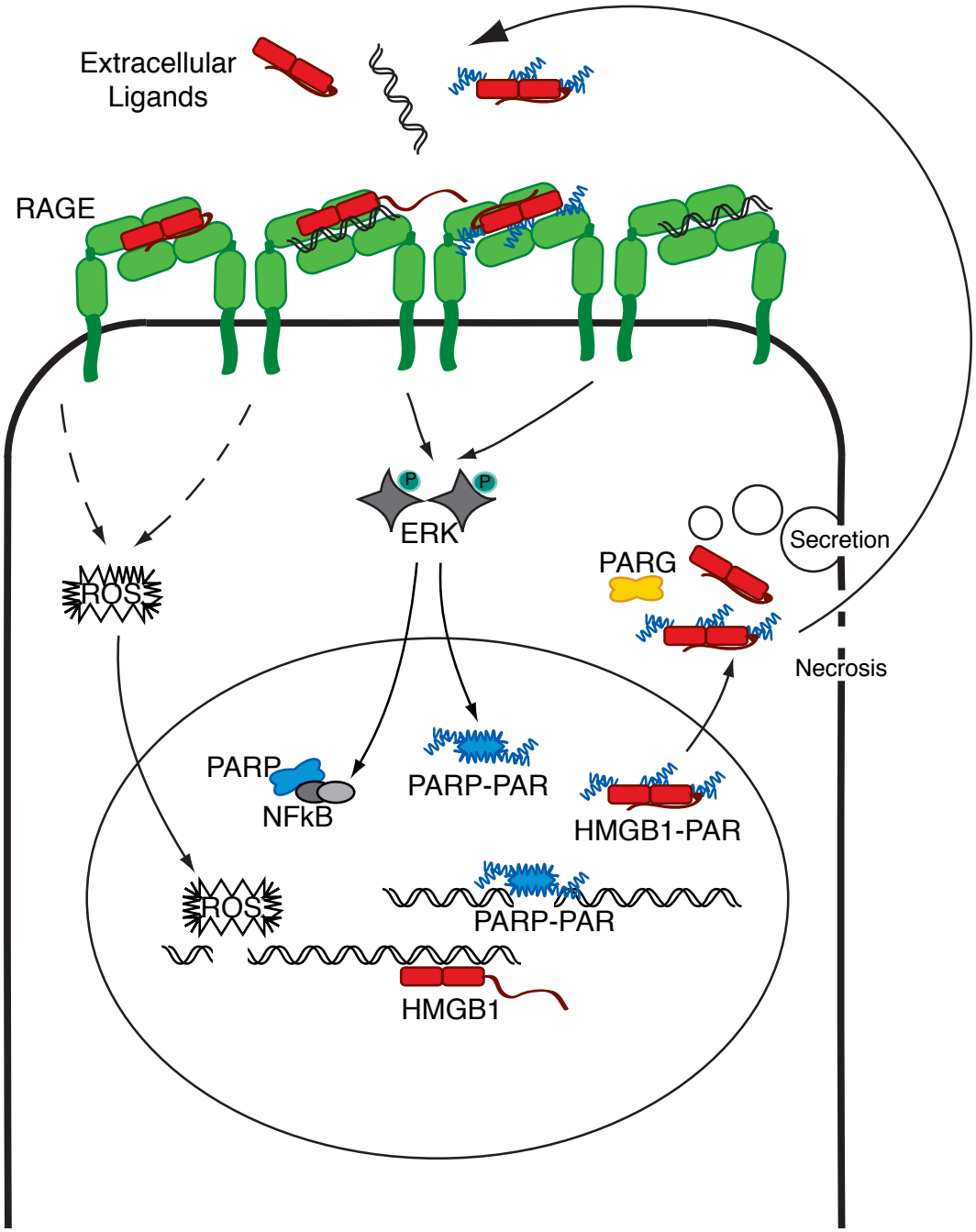


Figure 4.1 Model of the hypothetical interregulatory network formed by RAGE, HMGB1 and PARP.
For narration of the model and for explanation of abbreviations, please refer to the text and the List of Abbreviations.

these players contribute to modulation of cell stress responses and inflammation. Clearly defining the biology of these interactions may help to elucidate demonstrated connections between DNA damage, chronic inflammation, and cancer, as well as develop effective therapeutics that intervene in this network.

RAGE: a multifaceted modulator

The research presented here has focused on the manner in which RAGE interacts with nucleic acid-containing ligands, and the potential downstream consequences of these interactions on cell biology, inflammation and homeostasis. However, a survey of the RAGE literature indicates many distinct functions for RAGE, beyond that of a nucleic-acid receptor. While the interaction of RAGE with its other known ligands was discussed in Chapter I, several other functional roles for RAGE are worth acknowledging here:

1. **RAGE is important in development of the nervous system.** HMGB1 was initially identified as a RAGE ligand important in neurite outgrowth, the process by which developing neurons in the central nervous system begin to form axons and dendrites[110]. S100 proteins may induce similar effects in developing neurons and have been tied to growth of nervous system tumors[245]. RAGE also appears to aid in regeneration of damaged peripheral

nerves[246], thus supporting a role for this receptor in multiple aspects of neuronal growth. However, RAGE-deficient mice show no major developmental defects, neural or otherwise (refer to [247]), indicating that these roles in nervous system development are either redundant, or consist of subtle modulatory effects.

2. **RAGE is involved in cell adherence and spreading.** Expression of RAGE in HEK293 cells has been shown to increase the ability of these cells to adhere and spread on collagen IV-coated glass. Because collagen IV is a component of basement membranes[248] and the alveolar epithelial type-I cells that form alveolae highly express RAGE[140,249], this receptor may play an important role in the formation of alveolae during lung development (refer to [149]) or tissue repair. Interaction of cells with extracellular matrix is by no means limited to lung epithelia, and thus, RAGE may promote adherence in multiple cell types that express it.
3. **RAGE induces cytoskeletal rearrangement.** Cell growth, adherence and spreading mentioned in points 1 and 2 all require remodeling of the actin cytoskeleton. It is perhaps not surprising, then, that RAGE has been shown to associate with various proteins involved in cytoskeletal dynamics[119,137]. Demonstrated defects in formation of actin rings and podosomes in RAGE-deficient bone marrow precursor cells are a striking example, and these defects prevent proper development of bone-resorptive osteoclast cells.[250] As signaling and vesicular trafficking in the cytoplasm are all tied to the structural

framework of the cell, connections between cytoskeletal changes, RAGE ligand uptake, and RAGE signaling are likely to be an integrated and dynamic process.

4. **RAGE interacts with integrins.** RAGE has been reported to be a direct counter-receptor for the β2 integrin Mac-1 (CD11b)[127], and a key factor in leukocyte recruitment[251]. While interactions seem most likely to occur between Mac-1 on macrophages and RAGE on endothelia and epithelia, same-cell interactions of RAGE with αVβ3 integrins have also been suggested[250]. It is interesting to note that the two different RAGE dimer orientations described in Chapter II could account for these same-cell and inter-cell interaction modalities.

How might one reconcile the multiplicity of distinct RAGE functions with its role as a nucleic acid receptor? What is the advantage of having a single receptor interact with so many distinct ligands, and initiate multiple, distinct (and oft converging) signaling pathways?

I propose that RAGE serves as a clearinghouse for signal integration. Because of its broad ability to ligate negatively-charged molecules, it is an ideal integrator of multiple signals in a number of distinct tissue environments, including brain, lung and vasculature. The variety of roles attributed to RAGE may have more to do with the specializations of the cells by which it is expressed, than differences in receptor function itself. That is to say, in the

presence of growth signals, a neuron should grow. When in contact with the extracellular matrix, an epithelial or endothelial cell should adhere and spread. And in the presence of danger signals, an immune cell should respond. In the context of inflammation, RAGE seems to provide information about the type of threat that is perturbing homeostasis. Because of its location on the plasma membrane, RAGE senses that danger exists in the extracellular space. Detection of DNA in this environment indicates that self cells have been damaged or bacteria are present. Complexes of DNA with DAMPs like HMGB1 and LL37 indicate that a cellular response has already been initiated, thus releasing these molecules. The cellular response that led to formation of ligand complexes may be due to viral infection, and hence anti-viral interferon production is warranted. Production of cytokines and chemokines by “first responder” cells serves, in part, to recruit additional leukocytes and lymphocytes to the affected area. Thus, the ability to promote cytoskeletal rearrangement necessary for motility, as well as the ability of RAGE to simultaneously sense molecular patterns and interact with integrins on migrating cells, provides an efficient means of promoting response amplification. Interestingly, response downregulation is also built into RAGE function. Secretion of soluble RAGE (sRAGE) or cleavage of surface-expressed RAGE releases a decoy receptor into the extracellular milieu that can block the multiple effects of the membrane-bound receptor[252]. Upregulated release of sRAGE by epithelial cells upon cytokine stimulation has been shown in culture[119], and changes in sRAGE levels are

considered biomarkers in certain human diseases. Low levels of circulating sRAGE correlate with pathology in chronic inflammatory diseases like rheumatoid arthritis and atherosclerosis[252], suggesting that lack of blocking by sRAGE leads to greater inflammation. Paradoxically, high sRAGE levels have been found in SLE[252] and may represent an active, but inadequate, attempt to downregulate nucleic acid recognition pathways. Thus, RAGE may serve alternately as an amplifier and dampener of inflammatory responses, depending on the relative expression of its isoforms.

Perhaps the most important attribute for RAGE as a signal integrator is its regulability. Low constitutive expression in endothelia, epithelia and immune cells likely serves to keep RAGE among the very earliest sensors of perturbations in the environment. One could think of this as the “patrol officer” function of RAGE. Yet, RAGE may be most effective when it becomes upregulated in areas of ligand concentration. This could be considered the “riot police” function; large amounts of receptor are deployed to handle an increased disturbance in the environment. Just as having a high number of police on the streets all the time is a waste of resources and may lead to excessive martialism, high expression of a ligand-promiscuous, proinflammatory receptor is not something that would be of benefit under normal conditions. But in the same way riot police can help return a city to normalcy, so can expression of a master integrator of inflammatory signals, when the time is right.

This combination of regulability, site-specific expression, and promiscuity in both ligand binding and initiation of signaling cascades positions RAGE as an “ombudsman” molecule in cellular responses. It can sense the presence of key stimuli, influence their access to intracellular receptors, and either amplify or block signal generation, as the circumstances warrant. Although it is central in these processes, the role of RAGE is modulatory and often subtle, hence a lack of clear insufficiencies in RAGE knockout animals, and seemingly paradoxical disease phenotypes in both animals and humans. The ombudsman does not carry out all functions itself, but merely instigates, informs, promotes and suppresses according to the signals in its environment.

Future directions

In the context of healthy physiology, RAGE seems to be an effective modulator of inflammation and cell stress. The challenge appears when prolonged disturbances lead to chronic inflammation. Under these circumstances, RAGE becomes overwhelmed by excesses of ligands and amplification loops, and ceases to adequately regulate signals. Hence, specific blocking of RAGE could contribute positively to resolution of inflammation in diseases including diabetes, atherosclerosis, Alzheimer’s disease, some types of cancer, and SLE. Due to its natural ability to block membrane bound RAGE, sRAGE is a clear candidate for a

therapeutic agent; however, no clinical trials involving sRAGE administration have been published to date. Indirect methods of raising sRAGE levels, such as agents that promote cleavage of surface-bound RAGE by extracellular proteases, might be another means to achieve the desired blocking effect. Antibodies and peptides capable of directly blocking the ligand binding site(s) of RAGE may also be of therapeutic benefit. A RAGE-blocking compound, presumably of this type, is currently in clinical trial in the United States for treatment of Alzheimer's disease[253]. Intervention in receptor-mediated pathways can involve compounds that activate (agonists) as well those that block (antagonists). An application for RAGE agonists, rather than antagonists, seems less likely to have therapeutic potential in inflammatory disease. However, one could speculate that RAGE agonists might be beneficial in accelerating development, for instance in the lungs of premature infants, or in cases of peripheral nerve damage.

General immunosuppresive agents, such as corticosteroids, and general immune stimulators, such as IFN-α, often generate significant side effects in patients[254,255]. Design of modern therapeutics focuses on chemical and biological agents which can serve as "silver bullets" that target very specific inflammatory signaling axes, to achieve desired immune modulation without off-target effects. Hence, clarifying the multiplicity of roles for RAGE in ligand uptake, trafficking, and signaling will likely aid in this sort of cutting-edge drug design.

In terms of the nucleic acid - RAGE axis considered here, several key questions remain to be addressed. Identifying the specific cell types that mediate the phenotype of RAGE-dependent immune responses in the lung is a crucial first step. RAGE is expressed on both pulmonary epithelium and on immune cells. Expression of nucleic acid sensing TLRs is limited to a small subset of leukocytes, and hence, a role for RAGE in enhancing nucleic acid-TLR interaction is likely to be most relevant in these cell types. However, epithelial cell activation, characterized by the release of chemokines and expression of surface markers, plays an important role in immune cell recruitment. Defining the relative roles of these cell types when RAGE comes into contact with extracellular DNA is essential for dissecting how RAGE modulates immune responses that are ultimately dependent on activation of intracellular receptor proteins. Defining the relative role of distinct cell types in response to nucleic acids may also shed light on how RAGE intervenes in other inflammatory diseases that involve vascular pathology, such as atherosclerosis and diabetes.

Signals that originate directly from RAGE, in addition to those from classical nucleic acid sensors, also require further interrogation. MAP kinase and ERK phosphorylation, NF-κB activation, and cytokine release upon DNA stimulation of RAGE-expressing cells were addressed but not found in my studies; however, activation of these factors by RAGE upon ligand binding has been established clearly by others (see Chapter I). It is likely that cell type, relative expression level of RAGE isoforms and downstream signaling

intermediates, and other physiological factors influence RAGE signaling in response to a particular ligand. Defining the cell types responsible for RAGE-based responses to nucleic acids *in vivo* (as discussed above) may aid in determining the conditions required to assay direct RAGE signaling in response to nucleic acids in cell-based assays.

RAGE-enhanced activation of receptor axes other than TLRs are also an interesting avenue to pursue. As discussed earlier in this chapter, there is reason to suspect that nucleic acid-containing RAGE ligands may activate inflammasomes that regulate maturation of IL-1 family cytokines. IL-1 blockade is a broad therapeutic strategy employed for certain inflammatory conditions[256]. Due to the subtle nature of RAGE-based inflammatory modulation, preferential blockade of RAGE rather than IL-1 in inflammasome-related disorders may provide an attractive “silver bullet” approach.

The manner in which RAGE directly or indirectly interacts with the actin cytoskeleton is one of the least studied and perhaps one of the most interesting aspects of RAGE biology. In terms of interactions with nucleic acid sensors, understanding RAGE’s relationship with the cytoskeleton may shed light on the trafficking processes that bring intracellular sensors in contact with extracellular stimuli. The manner in which intracellular nucleic acid sensors “decide” to traffic between compartments such as the ER and endosomes is still largely unknown. It is attractive to speculate that RAGE, as a first-line receptor for nucleic acids on the cell surface, might initiate cytoskeleton-based intracellular trafficking of

sensor proteins to functional compartments. Understanding the specific manner in which RAGE integrates extracellular signals with intracellular structural changes may also clarify how molecular patterns induce cell motility and the tissue remodeling processes that lead to fibrosis and other forms of pathology.

In conclusion, the promiscuity and subtlety of RAGE make it both a central factor in receptor-mediated recognition of extracellular proinflammatory signals, and an integrator of multiple messages. As this body of work clearly defines RAGE as a nucleic-acid binding receptor, the implications of DNA and RNA interacting with RAGE add a novel set of pieces to the puzzle of how this protein detects homeostatic perturbations and promotes their resolution.

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