Published in final edited form as:

Biochem Biophys Res Commun. 2015 March 13; 458(3): 650–655. doi:10.1016/j.bbrc.2015.01.159.

Nuclear DAMP Complex-mediated RAGE-Dependent Macrophage Cell Death

Ruochan Chen^{a,b}, Sha Fu^b, Xue-Gong Fan^b, Michael T. Lotze^a, Herbert J. Zeh III^a, Daolin Tang^{a,*}, and Rui Kang^{a,*}

^aDepartment of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, USA

^bDepartment of Infectious Diseases and State Key Lab of Viral Hepatitis, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China

Abstract

High mobility group box 1 (HMGB1), histone, and DNA are essential nuclear components involved in the regulation of chromosome structure and function. In addition to their nuclear function, these molecules act as damage-associated molecular patterns (DAMPs) alone or together when released extracellularly. The synergistic effect of these nuclear DNA-HMGB1-histone complexes as DAMP complexes (nDCs) on immune cells remains largely unexplored. Here, we demonstrate that nDCs limit survival of macrophages (e.g., RAW264.7 and peritoneal macrophages) but not cancer cells (e.g., HCT116, HepG2 and Hepa1-6). nDCs promote production of inflammatory tumor necrosis factor α (TNF α) release, triggering reactive oxygen species-dependent apoptosis and necrosis. Moreover, the receptor for advanced glycation end products (RAGE), but not toll-like receptor (TLR)-4 and TLR-2, was required for Akt-dependent TNF α release and subsequent cell death following treatment with nDCs. Genetic depletion of RAGE by RNAi, antioxidant N-Acetyl-L-cysteine, and TNF α neutralizing antibody significantly attenuated nDC-induced cell death. These findings provide evidence supporting novel signaling mechanisms linking nDCs and inflammation in macrophage cell death.

Keywords

HMGB1; histone; DNA; RAGE; TNF α ; Akt

© 2015 Published by Elsevier Inc.

*Address correspondence and reprint requests to: Daolin Tang or Rui Kang, G.21, Hillman Cancer Center, 5117 Centre Ave, Pittsburgh PA, 15213, USA; Phone:+1-412-623-1211; Fax:+1-412-623-1212; tangd2@upmc.edu or kangr@upmc.edu.

Conflict of interest

All authors (Ruochan Chen, Sha Fu, Xue-Gong Fan, Michael T. Lotze, Herbert J. Zeh, III, Daolin Tang, and Rui Kang) declare no conflicts of interest or financial interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Damage-associated molecular pattern molecules (DAMPs) are endogenous molecules released from cells under stress, especially following injury or cell death [1]. They can act as inducers, sensors, and mediators of stress often promoting an innate and subsequent adaptive immune response through interaction with individual pattern recognition receptors [2]. DAMP receptors include toll-like receptors (TLRs) and the receptor for advanced glycation end products (RAGE) play important roles in human health and disease. Of particular importance are inflammatory and immune-associated diseases including both those due to infection and sterile inflammation. Many DAMPs are derived from the nucleus, and are thus collectively termed nDAMPs; these nDAMPs include high mobility group box 1 (HMGB1) [3,4] and components of the nucleosome (e.g., DNA [5] and histones [6]). In addition to exerting a singular effect, these nDAMPs usually are found as a complex to mediate inflammation and immune response [7,8] – which we more accurately refer to as nuclear DAMP complexes (nDCs). Thus, understanding the collective effects of nDCs on cells is important for devising treatment strategies for inflammation-associated disease.

The individual concentrations of HMGB1, histone, and DNA in the serum or plasma are simultaneously increased in patients with trauma, infection, autoimmune diseases, neurodegenerative diseases, and cancer [9,10,11]. Moreover, changes in their levels positively correlate with disease severity, progression, and prognosis. Of note, the average level of HMGB1, histone, and DNA in the circulation of these patients is usually measured in nanograms per milliliter (ng/mL) [12,13] whereas the quantities used in *in vitro* experimental studies usually measures in micrograms per milliliter (ug/mL) [14,15,16]. This is thus significantly higher than the concentrations found clinically. To more faithfully mimic the clinical setting, we initially evaluated the synergistic effects of HMGB1, histone, and DNA in combination in nDCs at low concentrations initially on the activity of macrophages. We provide here the first evidence that nDCs at low concentrations surprisingly induce macrophage cell death. Furthermore we demonstrate that such cell death is RAGE-mediated and oxidative stress dependent. These findings provide novel mechanisms linking nDAMPs and their complexes (nDCs) and regulation of the inflammatory response.

2. Methods

2.1 Regents

The antibodies to cleaved-PARP, P-Akt, LDH, LC3, and actin were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibodies to RAGE, TLR2, and TLR4 were obtained from Abcam (Cambridge, MA, USA). High purity HMGB1 protein was kindly provided by Dr. Jianhua Li from The Feinstein Institute for Medical Research (Manhasset, NY, USA) [17]. Mouse genomic DNA was obtained from New England BioLabs Inc. (Ipswich, MA, USA). High purity histone protein was obtained from Roche Life Science (Stockholm, Sweden). TNF α neutralizing antibody and control IgG were obtained from R&D Systems (Minneapolis, MN, USA). AKT inhibitor was obtained from Santa Cruz (Santa Cruz, CA, USA). ZVAD-FMK, necrostatin-1 and N-Acetyl-L-cysteine were obtained from Sigma (St. Louis, MO, USA).

2.2 Cell culture

The mouse macrophage cell line RAW264.7, human HCC cell line HepG2, mouse HCC cell line Hepa1-6, and human colorectal cancer cell line HCT116 were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained in Dulbecco's Modified Eagle's Medium or McCoy's 5a Medium Modified (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen) and 100ug/mL streptomycin (Invitrogen) and 100 U/mL penicillin (Invitrogen) in a humidified incubator with 5% CO₂ and 95% air.

2.3 Cell viability assay

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's instructions.

2.4 Cell clone formation assay

For all groups, 1 mL complete medium containing 500 cells were added to each well of a 12-well plate. Plates were incubated at 37 °C, 5 % CO₂ for 14 days. After that, cells were gently washed and stained with crystal violet. Colonies containing at least 50 cells were counted.

2.5 Western blot

Proteins in the cell lysate or supernatants were resolved on 4–12% Criterion XT Bis-Tris gels (Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane. After blocking, the membrane was incubated for two hours at 25°C or overnight at 4°C with various primary antibodies. After incubation with peroxidase-conjugated secondary antibodies for one hour at 25°C, the signals were visualized by enhanced or super chemiluminescence (Pierce, Rockford, IL, USA) according to the manufacturer's instruction. The relative band intensity was quantified using the Gel-pro Analyzer[®] software (Media Cybernetics, Bethesda, MD, USA).

2.6 RNAi

Specific RAGE-short hairpin RNA (shRNA), TLR2-shRNA, TLR4-shRNA, and control-shRNA were purchased from Sigma-Aldrich. Cells were seeded in six-well plates at a density of 5×10^5 cells/well to achieve a confluence of 70% overnight. The transfection was done using FuGENE[®] 6 Transfection Reagent (Roche) according to the manufacturer's instructions. The transfection efficiency by the shRNA was confirmed by Western blot.

2.7 Mice primary macrophages isolation

Peritoneal macrophages were isolated and cultured as previously described [18]. Described briefly, mice were i.p. injected with 2 ml of 4% thioglycollate. After three days of injection, peritoneal exudate cells were isolated by washing the peritoneal cavity with ice-cold Hank's Balanced Salt Solution (HBSS). These cells were incubated for two hours, and adherent cells were used as peritoneal macrophages.

2.8 Reactive Oxygen Species (ROS) assay

Fluorimetric analysis of ROS production was carried out using dye CH-H2DCFDA (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions.

2.9 TNF α release assay

TNF α release was assessed using a TNF α ELISA Assay Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.10 Statistical analyses

Data are expressed as means $\pm$ SD. Significance of differences between groups was determined by two-tailed Student's t test or ANOVA LSD test. A p-value < 0.05 was considered significant.

3. Results

3.1 DNA-HMGB1-histone complex (nDC) selectively inhibits macrophage cell viability in a TNF α -dependent manner

To observe the complex effects of HMGB1, histone, and DNA together at low doses on cell viability, we treated macrophages and cancer cells with exogenous HMGB1 (200 ng/mL), histone (300 ng/mL), and genomic DNA (500 ng/mL). Remarkably, there was a significantly combinatory effect of low dose HMGB1, histone, and DNA as a nDC in inhibiting cell viability at 24 hours and 48 hours in macrophages (RAW264.7 and primary mouse peritoneal macrophages), but not in cancer cells (HCT116, HepG2, and Hepa1-6) (Fig. 1A). Similarly, the long term clone forming ability of macrophage cell lines, but not cancer cell lines, was diminished following treatment with nDCs (Fig. 1B).

Tumor necrosis factor α (TNF α), a cytokine generated by immune cells, can cause cell death in response to inflammatory stimuli. Interestingly, TNF α release was increased at three and eight hours following nDC treatment in macrophages (RAW264.7), but not cancer cells (Hepa1-6) (Fig. 1C). Moreover, treatment with TNF α neutralizing antibody significantly increased macrophage (RAW264.7) viability in response to nDCs (Fig. 1D). Collectively, these findings suggest that neutralization of TNF α during the early stages can inhibit nDC-induced macrophage cell death.

3.2 TNF α -mediated oxidative stress facilitates nDC-induced apoptosis and necrosis in macrophages

According to morphological criteria, cell death is classified as apoptosis (type I), autophagy (type II), and necrosis (type III)[19]. We now know that autophagy as a stress response promotes cell survival under stress conditions [20] and only rarely is associated as a direct cause of cell death. To determine whether these cell death types are activated in response to nDCs, we monitored the expression of individual 'death markers' using Western blotting. Compared with individual component treatment, nDCs significantly increased the expression of cleaved poly ADP ribose polymerase (PARP, an apoptosis marker) and the release of lactate dehydrogenase (LDH, a necrosis marker) (Fig. 2A). In contrast, the expression of microtubule-associated protein light chain 3 (LC3)-I/II (an autophagy marker)

was decreased following treatment with nDCs (Fig. 2A). Moreover, Z-VAD-FMK ("ZVAD", a caspase inhibitor) and necrostatin-1 ("Nec-1", an inhibitor for programmed necrosis) partially decreased nDC-induced cell death (Fig. 2B). These findings suggest that nDCs induce both apoptosis and necrosis in macrophages.

Oxidative stress refers to elevated intracellular levels of reactive oxygen species (ROS) that is thought to be a critical common factor in the development of many types of cell death including apoptosis and necrosis [21]. The generation of ROS (monitored by analysis of CH-H2DCFDA) was increased in response to nDCs in RAW264.7 cells (Fig. 2C). In contrast, antioxidant N-Acetyl-L-cysteine (NAC) as well as TNF α neutralizing antibody significantly inhibited ROS production (Fig. 2B) and restored cellular viability (Fig. 1D and Fig. 2D) after treatment with nDCs. As expected, nDC-induced apoptosis (measuring cleaved PARP) and necrosis (measuring LDH release) were inhibited by treatment with NAC or TNF α neutralizing antibody (Fig. 2E). Collectively, these findings suggest that TNF α -mediated oxidative stress facilitates nDC-induced apoptosis and necrosis in macrophages.

3.3 RAGE is required for nDC-induced oxidative stress and macrophage cell death

nDAMPs are recognized by several cell surface receptors including RAGE and TLRs [22]. Interestingly, the expression of RAGE, but not TLR2 and TLR4, was upregulated at 24 hours following treatment with nDCs, suggesting a potential role of RAGE in the regulation of nDC-induced cell death (Fig. 3A). To further determine which receptor is required for nDC-induced macrophage cell death, we transfected RAW264.7 cells with specific shRNA targeting RAGE, TLR2, and TLR4, respectively (Fig. 3B). The knockdown of RAGE, but not TLR2 and TLR4, significantly attenuated nDC-induced ROS production (Fig. 3C) and subsequent cell viability (Fig. 3D). Similarly, nDC-induced apoptosis (measuring C-PARP) and necrosis (measuring LDH release) were inhibited after limiting expression of RAGE, but not TLR2 and TLR4. Thus, RAGE is required for nDC-induced oxidative stress and cell death in macrophages.

3.4 RAGE-mediated Akt activation is required for nDC-induced TNF α release in macrophages

As a serine/threonine kinase, Akt is activated through a phosphorylation mechanism. Akt activation appears to act as a dual regulator of inflammatory cytokine production in immune cells, depending on the nature of the stimulus [23]. To better understand the mechanisms of nDAMP-induced cell death in macrophages, we analyzed Akt activation in response to nDCs. Indeed, nDCs significantly induced Akt phosphorylation in macrophages (Fig. 4A). Knockdown of RAGE, but not TLR2 and TLR4, inhibited nDC-induced Akt phosphorylation in macrophages (Fig. 4A). Moreover, we demonstrated that both an Akt inhibitor (1,3-Dihydro-1-((4-(6-phenyl-1H-imidazo [4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidiny)-2H-benzimidazol-2-one trifluoroacetate salt hydrate) and knockdown of RAGE inhibited nDC-induced TNF α release in macrophages (Fig. 4B), suggesting a critical role for RAGE in the regulation of AKT activation and subsequent TNF α release in macrophages.

4. Discussion

Macrophages are central to the immune response to infection or accumulating damaged or dead cells. Their activity is regulated by both death and survival signaling pathways [24]. In this study, we provide the first evidence that nDAMP complexes (nDCs) consisting of DNA-HMGB1-histone, but not singular nDAMPs, can induce macrophage cell death through interaction with the pattern recognition receptor RAGE, but not TLR2 and TLR4. The activation of the RAGE signaling pathway may promote TNF α release, which triggers ROS-dependent apoptosis and necrosis. These findings therefore suggest a novel regulatory mechanism for controlling nDAMP activity in the innate immune response.

RAGE is a transmembrane immunoglobulin gene superfamily receptor, encoded within the major histocompatibility complex class III region. It is expressed by immune and non-immune cells and can interact promiscuously with multiple ligands including AGEs, HMGB1, S100, DNA, and RNA to activate the inflammatory pathway [25,26]. Dysfunction of RAGE signaling has been implicated in inflammation, metabolism, and certain diseases such as diabetes and cancer [27]. The function of RAGE in the regulation of cell survival and death is context-dependent. In cancer cells, overexpressed RAGE contributes to tumor survival. Knockout of RAGE *in vitro* or *in vitro* decreases tumor growth and metastasis [28,29,30,31], and increases chemotherapy sensitivity partly by limiting autophagy and increasing apoptosis [28]. In diabetic rat hearts, expression of RAGE promotes apoptosis after ischemic reperfusion [32]. Macrophages express high levels of RAGE. We found that knockdown of RAGE in macrophages suppressed nDC-induced apoptosis and necrosis. In contrast, TLR2 and TLR4 are not required for nDC-induced cell death. Collectively, these findings suggest that RAGE selectively mediates cell death in immune cells in response to nDCs. The structural nature of the nDCs requires further exploration.

Given the previous connections between oxidative stress and cell death observed in immune cells, we focused on determining whether ROS act as mediators of nDAMP complex-induced cell death. Our experiments demonstrated that ROS generation in macrophages is increased following treatment with nDCs. Antioxidant NAC supplementation reduces nDC-mediated apoptosis and necrosis in macrophages, nDAMPs are major regulator of NADPH oxidases in infection and sterile inflammation [33]. The interaction between the main cellular sources of ROS, such as mitochondria and NADPH oxidases, in response to nDCs, are currently not understood but likely involve both sources. In addition, RAGE-mediated oxidative stress determines the cellular/tissue fate in several diseases, such as myocardial ischemia, atherosclerosis, and diabetes [34,35,36]. Our study indicates that RAGE upregulation contributes to nDC-mediated oxidative injury in macrophages.

Importantly, we demonstrated that TNF α is a critical mediator of cell death upon activation of the nDC-RAGE signaling pathway. TNF α plays a critical role in cellular stress during infection and tissue damage. TNF α is not only released by activated macrophages, but also activates both cell-survival and cell-death mechanisms simultaneously [37,38]. TNF α -mediated caspase-8 activation is responsible for apoptosis [38]. TNF α also can trigger programmed necrosis (necroptosis) when caspase-8 activation is defective [39]. We found that the release of TNF α is increased in response to nDCs. Blocking TNF α activity with

neutralizing antibodies limits nDC-induced oxidative stress and cell death. We also found that activation of Akt by nDC-RAGE pathway contributes to TNF α release. Akt is a positive regulator of TNF α release in response to infection in macrophages and monocytes [40,41]. Inhibition of Akt significantly decreases proinflammatory cytokine production including TNF α in infected macrophages and monocytes [40,41]. In many cases, Akt activation promotes cell survival, whereas inactivation of Akt promotes apoptosis in a variety of cell types. In contrast, our findings indicate that Akt-mediated TNF α release promotes macrophage cell death, suggesting cell context modulates Akt function. Alternatively, Akt plays a different role in a variety of cellular functions depending on its phosphorylated substrates.

Collectively, we demonstrated that a complex of HMGB1, histone, and DNA at low-doses, so called nDCs, has a synergistic role in selectively triggering RAGE-dependent cell death in macrophages, but not cancer cells. RAGE is however overexpressed in many cancer cells [42], suggesting that RAGE is necessary but not sufficient to mediate nDC-induced cell death. Unraveling how this nDC regulates macrophage cell death will provide new therapeutic targets for treatment of inflammatory-associated diseases.

Acknowledgments

We thank Christine Heiner (Department of Surgery, University of Pittsburgh) for her critical reading of the manuscript. This work was supported by grants from the National Institutes of Health of USA (R01CA160417 to D.T.; R01CA181450 to H.J.Z/M.T.L) and the National Natural Sciences Foundation of China (81272253 to X.F.). This project used University of Pittsburgh Cancer Institute shared resources that are supported in part by award P30CA047904.

Abbreviations

DAMP	damage-associated molecular pattern
HMGB1	high mobility group box 1
TNFα	tumor necrosis factor α
RAGE	receptor for advanced glycation end products
TLR	toll-like receptor
NAC	N-Acetyl-L-cysteine
nDC	nuclear DAMP complex
PARP	poly ADP ribose polymerase
LDH	lactate dehydrogenase
LC3	microtubule-associated protein light chain 3
ROS	reactive oxygen species

References

1. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol.* 2007; 81:1–5. [PubMed: 17032697]

2. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. *Nat Rev Immunol.* 2009; 9:353–363. [PubMed: 19365408]
3. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature.* 2002; 418:191–195. [PubMed: 12110890]
4. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. HMG-1 as a late mediator of endotoxin lethality in mice. *Science.* 1999; 285:248–251. [PubMed: 10398600]
5. Marichal T, Ohata K, Bedoret D, Mesnil C, Sabatel C, Kobiyama K, Lekeux P, Coban C, Akira S, Ishii KJ, Bureau F, Desmet CJ. DNA released from dying host cells mediates aluminum adjuvant activity. *Nat Med.* 2011; 17:996–1002. [PubMed: 21765404]
6. Xu J, Zhang X, Pelayo R, Monestier M, Ammollo CT, Semeraro F, Taylor FB, Esmon NL, Lupu F, Esmon CT. Extracellular histones are major mediators of death in sepsis. *Nat Med.* 2009; 15:1318–1321. [PubMed: 19855397]
7. Liu L, Yang M, Kang R, Dai Y, Yu Y, Gao F, Wang H, Sun X, Li X, Li J, Cao L, Tang D. HMGB1-DNA complex-induced autophagy limits AIM2 inflammasome activation through RAGE. *Biochem Biophys Res Commun.* 2014; 450:851–856. [PubMed: 24971542]
8. Hou W, Zhang Q, Yan Z, Chen R, Zeh HJ III, Kang R, Lotze MT, Tang D. Strange attractors: DAMPs and autophagy link tumor cell death and immunity. *Cell death & disease.* 2013; 4:e966. [PubMed: 24336086]
9. Kang R, Chen R, Zhang Q, Hou W, Wu S, Cao L, Huang J, Yu Y, Fan XG, Yan Z, Sun X, Wang H, Wang Q, Tsung A, Billiar TR, Zeh HJ 3rd, Lotze MT, Tang D. HMGB1 in health and disease. *Mol Aspects Med.* 2014; 40C:1–116. [PubMed: 25010388]
10. Chen R, Kang R, Fan XG, Tang D. Release and activity of histone in diseases. *Cell death & disease.* 2014; 5:e1370. [PubMed: 25118930]
11. Farkas AM, Kilgore TM, Lotze MT. Detecting DNA: getting and begetting cancer. *Curr Opin Investig Drugs.* 2007; 8:981–986.
12. Kuroi K, Tanaka C, Toi M. Clinical significance of plasma nucleosome levels in cancer patients. *International Journal of Oncology.* 2001; 19:143–148. [PubMed: 11408935]
13. Pullerits R, Urbonaviciute V, Voll RE, Forsblad-D'Elia H, Carlsten H. Serum Levels of HMGB1 in Postmenopausal Patients with Rheumatoid Arthritis: Associations with Proinflammatory Cytokines, Acute-phase Reactants, and Clinical Disease Characteristics. *Journal of Rheumatology.* 2011; 38:1523–1525. [PubMed: 21724730]
14. Ries M, Schuster P, Thomann S, Donhauser N, Vollmer J, Schmidt B. Identification of novel oligonucleotides from mitochondrial DNA that spontaneously induce plasmacytoid dendritic cell activation. *Journal of Leukocyte Biology.* 2013; 94:123–135. [PubMed: 23610148]
15. Xu J, Zhang XM, Pelayo R, Monestier M, Ammollo CT, Semeraro F, Taylor FB, Esmon NL, Lupu F, Esmon CT. Extracellular histones are major mediators of death in sepsis. *Nature Medicine.* 2009; 15:1318–U1117.
16. Tang D, Kang R, Cheh CW, Livesey KM, Liang X, Schapiro NE, Benschop R, Sparvero LJ, Amoscato AA, Tracey KJ, Zeh HJ, Lotze MT. HMGB1 release and redox regulates autophagy and apoptosis in cancer cells. *Oncogene.* 2010; 29:5299–5310. [PubMed: 20622903]
17. Li J, Kokkola R, Tabibzadeh S, Yang R, Ochani M, Qiang X, Harris HE, Czura CJ, Wang H, Ulloa L, Warren HS, Moldawer LL, Fink MP, Andersson U, Tracey KJ, Yang H. Structural basis for the proinflammatory cytokine activity of high mobility group box 1. *Mol Med.* 2003; 9:37–45. [PubMed: 12765338]
18. Nomura F, Akashi S, Sakao Y, Sato S, Kawai T, Matsumoto M, Nakanishi K, Kimoto M, Miyake K, Takeda K, Akira S. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. *J Immunol.* 2000; 164:3476–3479. [PubMed: 10725699]
19. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nunez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon HU, Vandenabeele P, White E, Yuan J, Zhivotovskiy B, Melino G,

- Kroemer G. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ.* 2012; 19:107–120. [PubMed: 21760595]
20. Kroemer G, Marino G, Levine B. Autophagy and the integrated stress response. *Mol Cell.* 2010; 40:280–293. [PubMed: 20965422]
 21. Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suicide and survival. *J Cell Physiol.* 2002; 192:1–15. [PubMed: 12115731]
 22. Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. *Immunol Rev.* 2012; 249:158–175. [PubMed: 22889221]
 23. Lee YG, Lee J, Byeon SE, Yoo DS, Kim MH, Lee SY, Cho JY. Functional role of Akt in macrophage-mediated innate immunity. *Front Biosci (Landmark Ed).* 2011; 16:517–530. [PubMed: 21196185]
 24. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* 2008; 8:958–969. [PubMed: 19029990]
 25. Xie J, Mendez JD, Mendez-Valenzuela V, Aguilar-Hernandez MM. Cellular signalling of the receptor for advanced glycation end products (RAGE). *Cell Signal.* 2013; 25:2185–2197. [PubMed: 23838007]
 26. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, Avila C, Kambham N, Bierhaus A, Nawroth P, Neurath MF, Slattery T, Beach D, McClary J, Nagashima M, Morser J, Stern D, Schmidt AM. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell.* 1999; 97:889–901. [PubMed: 10399917]
 27. Sparvero LJ, Asafu-Adjei D, Kang R, Tang D, Amin N, Im J, Rutledge R, Lin B, Amoscato AA, Zeh HJ, Lotze MT. RAGE (Receptor for Advanced Glycation Endproducts), RAGE ligands, and their role in cancer and inflammation. *J Transl Med.* 2009; 7:17. [PubMed: 19292913]
 28. Kang R, Tang D, Schapiro NE, Livesey KM, Farkas A, Loughran P, Bierhaus A, Lotze MT, Zeh HJ. The receptor for advanced glycation end products (RAGE) sustains autophagy and limits apoptosis, promoting pancreatic tumor cell survival. *Cell Death Differ.* 2010; 17:666–676. [PubMed: 19834494]
 29. Taguchi A, Blood DC, delToro G, Canet A, Lee DC, Qu W, Tanji N, Lu Y, Lalla E, Fu C, Hofmann MA, Kislinger T, Ingram M, Lu A, Tanaka H, Hori O, Ogawa S, Stern DM, Schmidt AM. Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. *Nature.* 2000; 405:354–360. [PubMed: 10830965]
 30. Gebhardt C, Riehl A, Durchdewald M, Nemeth J, Furstenberger G, Muller-Decker K, Enk A, Arnold B, Bierhaus A, Nawroth PP, Hess J, Angel P. RAGE signaling sustains inflammation and promotes tumor development. *J Exp Med.* 2008; 205:275–285. [PubMed: 18208974]
 31. Heijmans J, Buller NV, Hoff E, Dihal AA, van der Poll T, van Zoelen MA, Bierhaus A, Biemond I, Hardwick JC, Hommes DW, Muncan V, van den Brink GR. RAGE signalling promotes intestinal tumorigenesis. *Oncogene.* 2012
 32. Bucciarelli LG, Ananthakrishnan R, Hwang YC, Kaneko M, Song F, Sell DR, Strauch C, Monnier VM, Yan SF, Schmidt AM, Ramasamy R. RAGE and modulation of ischemic injury in the diabetic myocardium. *Diabetes.* 2008; 57:1941–1951. [PubMed: 18420491]
 33. Tang D, Kang R, Zeh HJ 3rd, Lotze MT. High-mobility group box 1, oxidative stress, and disease. *Antioxid Redox Signal.* 2011; 14:1315–1335. [PubMed: 20969478]
 34. Yan SF, Ramasamy R, Schmidt AM. The RAGE axis: a fundamental mechanism signaling danger to the vulnerable vasculature. *Circ Res.* 2010; 106:842–853. [PubMed: 20299674]
 35. Naka Y, Bucciarelli LG, Wendt T, Lee LK, Rong LL, Ramasamy R, Yan SF, Schmidt AM. RAGE axis: Animal models and novel insights into the vascular complications of diabetes. *Arterioscler Thromb Vasc Biol.* 2004; 24:1342–1349. [PubMed: 15155381]
 36. Sbai O, Devi TS, Melone MA, Feron F, Khrestchatsky M, Singh LP, Perrone L. RAGE-TXNIP axis is required for S100B-promoted Schwann cell migration, fibronectin expression and cytokine secretion. *J Cell Sci.* 2010; 123:4332–4339. [PubMed: 21098642]
 37. Bradley JR. TNF-mediated inflammatory disease. *J Pathol.* 2008; 214:149–160. [PubMed: 18161752]

38. Wang L, Du F, Wang X. TNF- α induces two distinct caspase-8 activation pathways. *Cell*. 2008; 133:693–703. [PubMed: 18485876]
39. Linkermann A, Green DR. Necroptosis. *N Engl J Med*. 2014; 370:455–465. [PubMed: 24476434]
40. Rajaram MV, Ganesan LP, Parsa KV, Butchar JP, Gunn JS, Tridandapani S. Akt/Protein kinase B modulates macrophage inflammatory response to *Francisella* infection and confers a survival advantage in mice. *J Immunol*. 2006; 177:6317–6324. [PubMed: 17056562]
41. Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J Biol Chem*. 2002; 277:32124–32132. [PubMed: 12052830]
42. Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ. HMGB1 and RAGE in inflammation and cancer. *Annu Rev Immunol*. 2010; 28:367–388. [PubMed: 20192808]

Highlights

- Nuclear DAMP complexes (nDCs) selectively induce cell death in macrophages, but not cancer cells.
- TNF α -mediated oxidative stress is required for nDC-induced death.
- RAGE-mediated Akt activation is required for nDC-induced TNF α release.
- Blocking RAGE and TNF α inhibits nDC-induced macrophage cell death.

Figure 1. DNA-HMGB1-histone complex selectively inhibits macrophage cell viability in a TNF α -dependent manner

(A) Macrophages (RAW264.7 ["RAW"]) and primary mouse peritoneal macrophages ["MPM"]) and cancer cells (HCT116, HepG2, and Hepa1-6) were treated with HMGB1 (200 ng/mL), histone (300 ng/mL), genomic DNA (500 ng/mL), and nDC for 24 and 48 hours and cell viability was assayed (n=3, $P < 0.05$). (B) Analysis of long term clone forming ability of macrophages following treatment with DNA-HMGB1-histone complex ("DHH") as described in methods. (C) Analysis of TNF α release in RAW264.7 ["RAW"] and Hepa1-6 cells following treatment with DNA (500 ng/mL)-HMGB1 (200 ng/mL)-histone (300 ng/mL) complex ("DHH") for three-24 hours. (D) Analysis of cell viability in RAW264.7 ["RAW"] cells following treatment with DNA (500 ng/mL)-HMGB1 (200 ng/mL)-histone (300 ng/mL) complex ("DHH") for 24 hours with or without TNF α neutralizing antibody ("TNF α Ab", 500 ng/ml) or control IgG (n=3, $P < 0.05$).

Figure 2. TNF α -mediated oxidative stress facilitates nDC-induced apoptosis and necrosis in macrophages

(A) RAW264.7 cells were treated with HMGB1 (200 ng/mL), histone (300 ng/mL), genomic DNA (500 ng/mL) and DNA-HMGB1-histone complex for 24 hours, and the indicated protein levels were assayed using Western blot. (B) Analysis of cell viability in RAW264.7 cells following treatment with DNA (500 ng/mL)-HMGB1 (200 ng/mL)-histone (300 ng/mL) complex ("DHH") for 24 hours with or without ZVAD-FMK ("ZVAD", 20 μ M) and necrostatin-1 ("Nec-1", 5 μ M) (n=3, P < 0.05). (C-E) Analysis of CM-H2DCFDA fluorescence intensity (C), cell viability (D), and protein expression (E) in RAW264.7 cells following treatment with DNA (500 ng/mL)-HMGB1(200 ng/mL)-histone (300 ng/mL) complex ("DHH") for 24 hours with or without N-acetylcysteine ("NAC", 5mM) and TNF α neutralizing antibody ("TNF α Ab", 500 ng/ml) (n=3, P < 0.05).

Figure 3. RAGE is required for nDC-induced oxidative stress and macrophage cell death

(A) Western blot analysis of protein expression in RAW264.7 cells following treatment with DNA (500 ng/mL)-HMGB1 (200 ng/mL)-histone (300 ng/mL) complex (“DHH”) for indicated times. (B) Western blot analysis of protein expression in RAW264.7 cells after knockdown of indicated receptors. (C–E) Analysis of CM-H2DCFDA fluorescence intensity (C), cell viability (D), and protein expression (E) in indicated RAW264.7 cells following treatment with DNA (500 ng/mL)-HMGB1 (200 ng/mL)-histone (300 ng/mL) complex (“DHH”) for 24 hours (n=3, $P < 0.05$).

Figure 4. RAGE-mediated Akt activation is required for nDC-induced TNFα release in macrophages
(A) Western blot analysis of protein expression in indicated RAW264.7 cells following treatment with DNA (500 ng/mL)-HMGB1 (200 ng/mL)-histone (300 ng/mL) complex (“DHH”) for 24 hours. (B) Analysis of TNFα release in indicated RAW264.7 cells following treatment with DNA (500 ng/mL)-HMGB1 (200 ng/mL)-histone (300 ng/mL) complex (“DHH”) with or without AKT inhibitor (e.g., 1,3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidiny)-2H-benzimidazol-2-one trifluoroacetate salt hydrate, 10μM) for eight hours (n=3, P < 0.05).