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Mitochondrial Transcription Factor A Serves as a Danger Signal by Augmenting Plasmacytoid Dendritic Cell Responses to DNA

Mark W. Julian,*1 Guohong Shao,*1 Shengying Bao,* Daren L. Knoell,†*‡ Tracey L. Papenfuss,‡ Zachary C. VanGundy,‡ and Elliott D. Crouser*

Plasmacytoid dendritic cells (pDCs) are potent APCs known to regulate immune responses to self-Ags, particularly DNA. The mitochondrial fraction of necrotic cells was found to most potently promote human pDC activation, as reflected by type I IFN release, which was dependent upon the presence of mitochondrial DNA and involved TLR9 and receptors for advanced glycation end products. Mitochondrial transcription factor A (TFAM), a highly abundant mitochondrial protein that is functionally and structurally homologous to high mobility group box protein 1, was observed to synergize with CpG-containing oligonucleotide, type A, DNA to promote human pDC activation. pDC type I IFN responses to TFAM and CpG-containing oligonucleotide, type A, DNA indicated their engagement with receptors for advanced glycation end products and TLR9, respectively, and were dependent upon endosomal processing and PI3K, ERK, and NF-κB signaling. Taken together, these results indicate that pDC contribute to sterile immune responses by recognizing the mitochondrial component of necrotic cells and further incriminate TFAM and mitochondrial DNA as likely mediators of pDC activation under these circumstances.

The online version of this article contains supplemental material.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BAY, BAY 11-7085; CpGA, CpG-containing oligonucleotide, type A; CpGA-FFTC, FITC-labeled human CpGA; DAMP, danger-associated molecular pattern; EEA1, early endosome Ag1; F9L3L, F9L3 ligand; G-ODN, guanosine-rich inhibitory oligonucleotide; HMGB1, high mobility group box protein 1; IP, immunoprecipitation; IRF-7, IFN regulatory factor-7; mPDCA-1, mouse anti-pDC Ag1; pDC, plasmacytoid dendritic cell; RAGE, receptor for advanced glycation end product; sRAGE, soluble receptor for advanced glycation end product; TFAM, mitochondrial transcription factor A.

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important implications for the pathogenesis of sterile inflammatory responses in the context of clinical conditions associated with necrotic cell death and tissue damage.

Materials and Methods

Reagents

Human CpG-containing oligonucleotide, type A (CpGA, TLR9 ligand), mouse CpGA, FITC-labeled human CpGA (CpGA-FITC), guanosine-rich inhibitory oligonucleotide (G-ODN, TLR9 signaling inhibitor), an inhibitor of endosomal acidification (chloroquine), HEK-Blue hTLR2 cells, and Quanti-Blue were obtained from InvivoGen (San Diego, CA). Cyclosporin A, Boc-Phe-Leu-Phe-Leu-Phe, and Trp-Arg-Trp-Trp-Trp-Trp were acquired from Alexon (San Diego, CA), ChemPep (Miami, FL), and Toxor Bioscience (Bristol, U.K.), respectively. BAY 11-7085 (BAY) and heparin sodium were obtained from Calbiochem (EMD Biosciences, Rockland, MA) and APP Pharmaceuticals (Schaumburg, IL) respectively. Primary Abs were acquired commercially: transcriptional regulator, IFN regulatory factor-7 (IRF-7), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) and an endosomal marker, early endosome Ag 1 (EEA1), p-Akt, p-ERK, and p-NF-κB (Cell Signaling Technology, Danvers, MA).

Table I. Primer sequences used for PCR

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<tr>
<th>Gene</th>
<th>Amplicon (bp)</th>
<th>Sense/Antisense</th>
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<td>Sense</td>
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<td>NADH 6</td>
<td>52</td>
<td>Sense/Antisense</td>
<td>5′-CCAGAAGACACCCACCCACCG-3′</td>
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</table>
Immunofluorescent Abs, DAPI, Alexa Fluor 488, and Alexa Fluor 555 were obtained from Molecular Probes (Invitrogen, Carlsbad, CA). Anti-human TFAM polyclonal Ab was made by Spring Valley Laboratories (Woodbine, MD). The epitope used to create the Ab was KQRKYG, which was chosen because the corresponding peptide sequence was unique relative to that of HMGB1. The anti-human TFAM Ab was shown to be selective for TFAM (Supplemental Fig. 2). The HMGB1 blocking Ab was provided by Dr. K.J. Tracey (Feinstein Institute for Medical Research, Manhasset, NY). Secondary Abs were obtained from Cell Signaling Technology, and PI3K inhibitors LY294002 and wortmannin were acquired from Cell Signaling Technology and Invitrogen, respectively. IL-3, IL-4, IL-6, IL-10, and IL-12 (10 ng/ml), IL-12p40 (10 ng/ml), and IL-12p70 (10 ng/ml) were obtained from R&D Systems (Minneapolis, MN). HepG2 and HEK-293 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured using DMEM/F-12 and MEM (Invitrogen), respectively. Unless otherwise stated, all additional chemicals were obtained from Sigma-Aldrich (St. Louis, MO) using the best available grade.

**Plasmid construction and purification of recombinant proteins**

Recombinant TFAM was synthesized as described in detail previously (5). In brief, the coding region of human TFAM was amplified by PCR, cloned into pcDNA3.1*Inv*-His A (Invitrogen), and pDsRed-Express-C1 (BD Clontech, Mountain View, CA) vectors in-frame or with added C-terminal myc epitope and 6× histidine tags and then transformed into Escherichia coli competent cells (DH5α; Invitrogen). The resultant TFAM plasmids, pcDNA3.1-TFAMmyc0.6×His and pDsRed-TFAM-C1, were transfected into HEK-293 cells, and the resultant polyhistidine-tagged recombinant proteins (TFAM and DsRed-TFAM) were then purified by Ni-NTA nickel-chelating resin. Likewise, the coding region for human RAGE comprising only the extracellular domain was cloned into the pcDNA3.1*Inv*-His A vector and processed similarly to obtain recombinant soluble RAGE (sRAGE). The coding region of human RAGE (GenBank accession number BC020669 [www.ncbi.nlm.nih.gov/gene/177]) was amplified by PCR using the pDNR-LIB-RAGE template (American Type Culture Collection). The sense primer for human sRAGE (5′-CGAATTCCCACCAT-ACCAGTTCCCAGCCCTGATCC-3′) introduced an EcoRI restriction site to facilitate cloning. The antisense primer for human sRAGE (5′-TGGTGACCAGTACCAGACTCAGGCCTACAGGCC-3′) introduced a KpnI restriction site (13–14). The identity and function of the recombinant proteins were confirmed by Western blot analysis, capillary-liquid chromatography-nano-spray tandem mass spectrometry, and the EMSA (5). HMGB1 was synthesized and confirmed in a similar manner as detailed previously (5) for use in determining Ab cross-reactivity.

**HepG2 subcellular fractionation**

HepG2 cell necrosis was induced by freeze/thaw and was confirmed by the lactate dehydrogenase assay, as described previously (5, 15). Cell lysates, along with nuclear, cytoplasmic, and total mitochondrial protein cellular subfractions, were prepared from HepG2 cells using a differential centrifugation approach (16) with minor modifications. Genomic and mitochondrial DNA were removed, when indicated, using benzonase (Novagen, San Diego, CA). Great care was taken to ensure purity of the cellular subfraction proteins, which was further confirmed by Western blot analysis using Abs against markers specific for plasma membrane (CEACAM-1), endoplasmic reticulum (calnexin), and mitochondria (COXII and HSP60) (5). The endotoxin level in each cellular subfraction protein was determined using the chromogenic Limulus amebocyte lysate assay (Associates of Cape Cod, East Falmouth, MA). When needed, samples were passed through AffinityPak Detoxi-Gel columns (Pierce Biotechnology, Rockford, IL), according to the manufacturer’s recommendations, and restested by Limulus amebocyte lysate to confirm that endotoxin levels were ≤ 5 pg/μg protein. The absence of Mycoplasma contamination was confirmed by PCR (see methodology below and Table I for primers used) and by treating HEK-Blue hTLR2 cells (HEK-293 cells stably coexpressing a human TLR2 gene and a NF-κB-inducible SEAP reporter gene that can be easily monitored using secreted embryonic alkaline phosphatase detection media [Quanti-Blue], according to the manufacturer’s recommendations) with the isolated protein cellular subfractions, the recombinant proteins, and the media bathing the cells (i.e., supernatants) from which the proteins were isolated or synthesized, respectively (Supplemental Fig. 1).

**Isolation, culture, and stimulation of human pDC**

Normal peripheral blood pDC were isolated from healthy human blood donors (n = 20) according to approved university Institutional Review Board guidelines (including signed written informed consent) using the MACS CD304 (BDCA-4/neuropilin-1) MicroBead positive selection kit into pcDNA3.1*Inv*-His A (Invitrogen), and pDsRed-Express-C1 (BD Clontech, Mountain View, CA) vectors in-frame or with added C-terminal myc epitope and 6× histidine tags and then transformed into Escherichia coli competent cells (DH5α; Invitrogen). The resultant TFAM plasmids, pcDNA3.1-TFAMmyc0.6×His and pDsRed-TFAM-C1, were transfected into HEK-293 cells, and the resultant polyhistidine-tagged recombinant proteins (TFAM and DsRed-TFAM) were then purified by Ni-NTA nickel-chelating resin. Likewise, the coding region for human RAGE comprising only the extracellular domain was cloned into the pcDNA3.1*Inv*-His A vector and processed similarly to obtain recombinant soluble RAGE (sRAGE). The coding region of human RAGE (GenBank accession number BC020669 [www.ncbi.nlm.nih.gov/gene/177]) was amplified by PCR using the pDNR-LIB-RAGE template (American Type Culture Collection). The sense primer for human sRAGE (5′-CGAATTCCCACCAT-ACCAGTTCCCAGCCCTGATCC-3′) introduced an EcoRI restriction site to facilitate cloning. The antisense primer for human sRAGE (5′-TGGTGACCAGTACCAGACTCAGGCCTACAGGCC-3′) introduced a KpnI restriction site (13–14). The identity and function of the recombinant proteins were confirmed by Western blot analysis, capillary-liquid chromatography-nano-spray tandem mass spectrometry, and the EMSA (5). HMGB1 was synthesized and confirmed in a similar manner as detailed previously (5) for use in determining Ab cross-reactivity.

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**IFN-α measurements**

Human pDC or mouse splenocyte (see below) supernatants, collected ~24 h posttreatment, were analyzed for their IFN-α (R&D Systems) concentrations by ELISA, according to the manufacturer’s recommendations.

**Immunoprecipitation, PCR, and Western blotting**

Necrotic HepG2 cell (~7 × 10⁶/ml) lysates induced by freeze/thaw (5, 15) underwent immunoprecipitation (IP) designed to pull-down native TFAM protein (IP TFAM). After the addition of protease inhibitors (1 mM PMSF, 5, 15) and centrifuging at 1500 g, the supernatant was incubated with rabbit anti-human TFAM primary Ab (1:500). Pure human recombinant TFAM (0.5 µg) was used as a positive control. TFAM protein (0.5 µg/ml) or CpGA DNA (1 µM) alone or in combination for 1.5 h. At the end of timed treatment, cells were treated depending upon the experiment being performed. To examine nuclear translocation of IRF-7, cells were incubated with DsRed-TFAM (5 µg/ml) or CpGA DNA (1 µM) alone or in combination for 3 h. Alternatively, to examine endosomal colocalization, cells were incubated with DsRed-TFAM (5 µg/ml) or CpGA-FITC DNA (1 µM) alone or in combination for 1.5 h. At the end of timed treatment, cells were washed twice with PBS and then fixed with 1% paraformaldehyde. Cell suspensions were then applied to slides for further staining using cytospin. Following blockade with 5% goat serum/1% FBS in PBS for 1 h at room temperature, immunofluorescent staining of fixed pDC was carried out according to experimental design.

**Confocal fluorescence microscopy**

Normal human pDCs were isolated from peripheral blood by positive selection as detailed above and resuspended (at 1 × 10⁵ cells/ml) in X-Vivo 15 media with 1% FBS and 25 ng/ml IL-3 at 37°C in a 5% CO2-humidified atmosphere. After 1 h, cells were treated depending upon the experiment being performed. To examine nuclear translocation of IRF-7, cells were incubated with TFAM (5 µg/ml) or CpGA DNA (1 µM) alone or in combination for 3 h. Alternatively, to examine endosomal colocalization, cells were incubated with DsRed-TFAM (5 µg/ml) or CpGA-FITC DNA (1 µM) alone or in combination for 1.5 h. At the end of timed treatment, cells were washed twice with PBS and then fixed with 1% paraformaldehyde. Cell suspensions were then applied to slides for further staining using cytospin. Following blockade with 5% goat serum/1% FBS in PBS for 1 h at room temperature, immunofluorescent staining of fixed pDC was carried out according to experimental design.

**FIGURE 2.** Mitochondrial DNA is uniquely associated with TFAM when released from necrotic HepG2 cells, which can subsequently induce pDC type I IFN activation. The presented data were representative of three independent preparations. (A) Necrotic HepG2 cell lysates subjected to IP with a TFAM Ab, yielded IP TFAM protein (Western blot, left panel) that is bound to DNA (~120 ng/µl protein) that has the “unique” gene expression profile of mitochondrial DNA (post-PCR cDNA gel, right)—8 of 13 mitochondrial genes demonstrated along with 1 nonmitochondrial [COX IV] (lane 1, NADH 1; lane 2, NADH 4; lane 3, NADH 6; lane 4, COX I; lane 5, COX II; lane 6, COX III; lane 7, COX IV; lane 8, cytochrome b; and lane 9, ATP synthase 6]). (B) Exposure of purified human pDCs (1 × 10⁵ cells/ml) to IP TFAM (0.5 µg/ml) yielded marked release of IFN-α by 24 h posttreatment (*p < 0.01, compared with no treatment).
FIGURE 3. TFAM amplifies CpGA DNA-induced pDC IFN-α release, which is dependent upon RAGE and endosomal processing. The presented data were derived from or is representative of at least five independent experiments. (A) CpGA DNA (0.5 μM)-induced pDC (1 × 10^5 cells/ml) IFN-α release was significantly augmented by coincubation with human recombinant TFAM (5 μg/ml) 24 h posttreatment. This effect was dramatically attenuated through RAGE (20 μg/ml sRAGE) or TLR9 (10 μM G-ODN) inhibition (*p < 0.01, relative to no treatment, †p < 0.01, compared with CpGA DNA alone, ‡p < 0.01, relative to no treatment and the TFAM + CpGA treatment group). (B) Twenty-four hours posttreatment, heparin notably inhibited TFAM + CpGA DNA-induced pDC IFN-α release in a dose-dependent manner (*p < 0.01, compared with no treatment, †p < 0.05, relative to no treatment and the TFAM + CpGA treatment group, ‡p < 0.01, compared with the TFAM + CpGA treatment group). (C) Representative fluorescent photomicrographs demonstrating colocalization of recombinant human TFAM (TFAM-DsRed) and CpGA DNA (CpGA-FITC) and colocalization of CpGA DNA (CpGA-FITC) with endosomes (EEA1) within pDCs at 1.5 h posttreatment (original magnification ×40). (D) TFAM + CpGA DNA-induced IFN-α release was completely prevented at 24 h posttreatment by inhibiting endosomal acidification with chloroquine (100 μM CLQ) pretreatment (*p < 0.01, relative to no treatment, †p < 0.01, compared with CpGA DNA alone, ‡p < 0.01, relative to the TFAM + CpGA treatment group).
For IRF-7 nuclear translocation, cells were incubated with rabbit anti-human IRF-7 Ab (1:100) overnight at 4°C and then followed by goat anti-rabbit Alexa Fluor 488-labeled secondary Ab (1:500) for 1 h at room temperature. Cell nuclei were stained with propidium iodide (50 ng/ml; BD Biosciences, San Jose, CA) for 10 min. Positive translocation of IRF-7 was observed to change pDC nuclei from red to yellow in color. This was quantified by determining the number of positive cells as a percentage of the total cell count in a minimum of 10 high-power fields (×40) for each of three experimental preparations for each treatment group. For endosomal colocalization, cells were incubated with rabbit anti-human EEA1 Ab (1:100) overnight at 4°C and then followed by either goat anti-rabbit Alexa Fluor 488-labeled (when staining in conjunction with DsRed-TFAM treatment) or Alexa Fluor 555-labeled (when staining in conjunction with CpGA-FITC DNA treatment) secondary Abs (1:500) for 1 h at room temperature. In this case, cell nuclei were stained with DAPI (100 ng/ml) for 10 min. Immunofluorescence was then evaluated using confocal fluorescence microscopy.

Mouse pDC expansion and splenocyte preparation

All experiments were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee in accordance with National Institutes of Health guidelines. Eight-week-old, adult, male, C57BL/6 mice (~25 g) (The Jackson Laboratory, Bar Harbor, ME) were used for in vivo pDC expansion and as a source for splenocytes. pDC expansion was carried out using melanoma cells (expressing murine [Flt3]) as described previously (18, 19). Briefly, 4 × 10^6 B16 melanoma cells (C57BL/6 background), transfected with mouse recombinant Flt3 ligand (Flt3L) cDNA using a murine leukemia retroviral vector, were suspended in sterile saline and injected s.c. in two sites over both flanks. Mice were euthanized when the tumor size reached 1–1.5 cm in diameter (after 3–4 wk) (18), and the spleens were harvested.

**FIGURE 4.** Increased pDC IFN-α transcript and protein release induced by CpGA DNA or TFAM + CpGA DNA treatment involves PI3K and IRF-7 signaling. The presented data were derived from or is representative of at least five independent experiments. (A) Both CpGA DNA and TFAM + CpGA DNA-induced pDC IFN-α release was completely inhibited at 24 h posttreatment following LY294002 (5 μM LY) or wortmannin (1 μM Wort) pretreatment (*p < 0.01, compared with no treatment; †p < 0.01, relative to CpGA DNA alone; ‡p < 0.01, compared with the CpGA or TFAM + CpGA treatment groups). (B) Representative relative gene expression in pDCs as determined by real-time PCR was increased for all subtypes of IFN-α (nearly three orders of magnitude) and of IRF-7 following TFAM + CpGA DNA treatment at ~4 h posttreatment. This increased expression was markedly reduced (nearly two orders of magnitude for all IFNα subtypes) with LY294002 (5 μM LY) pretreatment.

Single-cell splenocyte suspensions were prepared following mechanical disaggregation of the spleen tissue and gently passing the released cells and tissue fragments through a 70-μm nylon cell strainer. Following erythrocyte lysis, cells were washed and then suspended in RPMI 1640 medium (supplemented with 25 mM HEPES, 2 mM l-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% FBS) (Invitrogen). Cells were then cultured in 48-well plates at a concentration of 1 × 10^6/ml in the above media with only 2% FBS at 37°C in a 5% CO₂-humidified atmosphere. Polymyxin B (10 μg/ml) was routinely added to the cells in each well to further block the effect of any possible endotoxin contamination. After 1 h, desired concentrations of CpGA DNA and TFAM (alone or in combination) were then added to the medium. All pre-incubations with inhibitors of RAGE (20 μg/ml sRAGE and 1–100 U/ml heparin sodium), TLR9 (10 μM G-ODN), endosomal acidification (100 μM chloroquine), P3K signaling (5 μM LY294002 and 1 μM wortmannin), NF-κB signaling (5 μM BAY), or HMGB1 blocking Ab (5 μg/ml) were made as a one-time dose 30 min prior to TFAM and/or CpGA DNA addition. The cell supernatants were then collected at ~24 h posttreatment and stored at −80°C for later analyses. Similar experiments involving treatment with CpGA DNA and TFAM (alone or in combination) were carried out in mouse splenocytes isolated from matched RAGE knockout (RAGE^−/−) mice (provided by Drs. A. Bierhaus and P.P. Nawroth) wherein RAGE depletion was confirmed by real-time PCR. Relative copy numbers and expression ratios for the various RAGE-associated gene loci were normalized to the expression of the housekeeping gene, β-actin (see Table I for primers used, Supplemental Fig. 3).

Elevated pDC populations following Flt3L expansion were identified via flow cytometry following immunofluorescent staining of splenocytes with mouse anti-pDC Ag I (mPDCA-1)-FITC (Milenyi Biotec).
Signaling in mouse splenocytes

PI3K/Akt, ERK, and NF-κB signaling in mouse splenocytes was investigated following their treatment with CpGA DNA (0.3 μM) alone or in combination with TFAM (5 μg/ml). Cells were collected at 15, 30, and 60 min posttreatment and processed for Western blot analyses according to standard techniques. Possible inhibition of these signaling pathways was further examined by pretreatment with sRAGE (20 μg/ml), LY294002 (5 μM), or BAY (5 μM) for 30 min. The rabbit polyclonal primary Abs used were p-Akt (1:500), p-ERK (1:1000), and p-NF-κB (1:1000). Protein band densities were normalized to protein load established by subsequent use of mouse monoclonal β-actin Ab (1:5000) and compared with corresponding bands from untreated cells.

Statistical analyses

The data were derived from independent experiments, as designated in the figure legends, and was expressed as mean ± SEM, and statistical significance was based on a p value ≤ 0.05. SigmaPlot 12.0 and SYSTAT 13.0 software were used to plot the data and carry out the statistical analyses, respectively. pDC IFN-α release in response to various treatments were compared with untreated controls using the Wilcoxon rank-sum test. Additional comparisons involving pretreatment inhibition studies and all experiments relating multiple groups were made using the Kruskal–Wallis test. Where appropriate, post hoc analyses between group rank means were performed using Dunn’s test.

Results

Selective activation of pDCs by mitochondrial Ags involved TLR9 and RAGE

Previous studies have demonstrated that nuclear HMGB1 (1, 10, 15), and various mitochondrial Ags (2–6), are potent endogenous danger signals. To determine which component of necrotic cells was most immunogenic, pDCs were exposed to equal concentrations of purified cellular fractions derived from necrotic HepG2 cells. pDCs were observed to respond most vigorously in terms of type 1 IFN release to the mitochondrial component of the cell (Fig. 1A), and this response was largely dependent upon the presence of mitochondrial DNA (Fig. 1B). An essential role of TLR9 was confirmed by the significant attenuation of pDC activation by mitochondrial Ags in the presence of G-ODN, a competitive TLR9 inhibitor (Fig. 1C). In keeping with previous studies implicating RAGE agonists as important danger signals in the setting of cell death (1, 10), pretreatment with sRAGE notably inhibited mitochondrial Ag-mediated pDC activation (Fig. 1C). TFAM, a high-abundance mitochondrial protein normally bound to mitochondrial DNA (12) that is released from necrotic cells (5), was shown to remain associated with mitochondrial DNA upon IP following necrotic cell death (Fig. 2A), and treatment with this IP TFAM induced significant pDC IFN-α release (Fig. 2B). This observation led to the consideration that TFAM may modify pDC activation by DNA.

TFAM augmented pDC activation through RAGE and endosomal processing

HMGB1, a potent RAGE agonist, is known to promote pDC activation by facilitating the association of TLR9 receptors and agonists within endosomes (9). Given that TFAM is normally bound to mitochondrial DNA, thereby preventing DNA degradation (12) and is homologous in structure and function to HMGB1 (11), we tested the hypothesis that TFAM augments pDC activation by CpGA DNA, a potent TLR9 agonist, through a RAGE-dependent mechanism. As presented in Fig. 3A, treatment with highly purified human recombinant TFAM significantly enhanced IFN-α release in the presence of CpGA DNA, and this synergistic effect was attenuated in the presence of sRAGE or the TLR9 inhibitor G-ODN. Like sRAGE, RAGE–ligand inhibition was further demonstrated wherein pretreatment with heparin decreased pDC IFN-α release in a dose-dependent manner (Fig. 3B). Interestingly, there was no detectable effect upon TFAM plus CpGA-induced pDC type 1 IFN activation by formyl peptide receptor inhibition (Supplemental Fig. 4). In keeping with previous investigations showing that Ag-dependent TLR9 signaling in

FIGURE 5. TFAM + CpGA DNA-induced nuclear translocation of IRF-7 in pDCs occurs independently of PI3K signaling. The presented data were derived from or is representative of at least five independent experiments. (A) Representative fluorescent photomicrographs of nuclear translocation of IRF-7 in pDCs in untreated (left panel), TFAM + CpGA DNA-treated (middle panel), and TFAM + CpGA DNA-treated following LY294002 (5 μM) pretreatment (right panel) conditions 3 h posttreatment (black arrows, negative cells; blue arrows, positive cells) (original magnification X40). Positive translocation of IRF-7 (green) was observed when pDC nuclei changed from red to yellow in color. (B) No significant change (positive cell count as a percentage of the total per high-power field [HPF]) was determined in the TFAM + CpGA DNA-induced increase in nuclear translocation of IRF-7 following LY294002 (5 μM LY) pretreatment (*p < 0.01, relative to no treatment, †p < 0.05, compared with CpGA DNA alone).
pDCs occurred within endosomes (20), fluorescence confocal microscopy confirmed early colocalization of TFAM and CpGA DNA within pDC endosomes (Fig. 3C). Dramatic inhibition of pDC responses to TFAM plus CpGA DNA by pretreatment with an inhibitor of endosomal acidification (chloroquine) confirmed the essential role of endosomal signaling for pDC TLR9-dependent IFN-α production (Fig. 3D).

PI3K was essential for pDC type I IFN responses to TFAM and CpGA DNA

Guiducci et al. (21) recently reported that PI3K regulates the transcription of IFN-α by facilitating nuclear translocation of IFN regulatory factor-7 (IRF-7). Fig. 4A provides evidence that PI3K was essential for activation of pDCs by TFAM and CpGA DNA wherein PI3K inhibition (LY294002 and wortmannin) completely

**FIGURE 6.** TFAM amplifies CpGA DNA-induced IFN-α release in mouse splenocytes, which is dependent upon the pDC population size, RAGE, TLR9, endosomal processing, and PI3K signaling. The presented data were derived from or is representative of at least five independent experiments. (A) Flow cytometry results demonstrating the increase in the pDC-positive (as indicated using the specific pDC marker, mPDCA-1) cell population in mouse splenocytes following exposure to B16 melanoma cell-expressed murine Flt3L. Expansion was observed to routinely double pDC populations after the 3- to 4-wk exposure (note: percent positive cell count increase at M1). (B) Mouse CpGA (mCpGA) DNA (0.3 μM)-induced IFN-α release was increased by human recombinant TFAM (5 μg/ml) coinoculation in mouse splenocytes (1 × 10^6 cells/ml) 24 h posttreatment. This effect was significantly attenuated through RAGE (20 μg/ml sRAGE), TLR9 (10 μM G-ODN), endosomal acidification (100 μM chloroquine [CLQ]), PI3K signaling (5 μM LY), or NF-κB signaling (5 μM BAY) inhibition (*p < 0.01, relative to no treatment, †p < 0.01, compared with CpGA DNA alone, ‡p < 0.01, relative to the TFAM + CpGA treatment group). Pretreatment with HMGB1 blocking Ab had no effect upon TFAM + CpGA DNA-induced splenocyte IFN-α release. Notably elevated splenocyte IFN-α release was demonstrated in splenocytes subjected to Flt3L-induced expansion consistent with increased pDC cell populations. (C) Inhibition of TFAM + CpGA DNA-induced splenocyte type I IFN activation by heparin was dose dependent (*p < 0.01, compared with no treatment, †p < 0.05, relative to no treatment and the TFAM + CpGA treatment group, ‡p < 0.01, compared with the TFAM + CpGA treatment group). (D) Splenocytes from RAGE−/− mice demonstrated significantly diminished IFN-α release in response to CpGA DNA treatment alone or in combination with TFAM (*p < 0.01, relative to no treatment, †p < 0.05, compared with no treatment and CpGA DNA alone, ‡p < 0.05, relative to CpGA DNA alone, †p < 0.01, compared with the TFAM + CpGA treatment group).
blocked Ag-induced IFN-α release. Likewise, NF-κB inhibition (BAY) proved equally effective at preventing Ag-induced pDC type I IFN activation (data not shown). As previously reported and in contrast to other TLR ligands (22), TLR9-dependent pDC activation by TFAM and CpGA DNA promoted the transcription of all IFN-α subtypes. Moreover, the expression of all IFN-α subtype transcripts depended upon PI3K signaling (Fig. 4B). In contrast to the earlier study (16), PI3K signaling minimally influenced IRF-7 nuclear translocation, as reflected by fluorescence microscopy (Fig. 5).

Murine splenocyte responses mirror-isolated human pDC responses to TFAM and CpGA DNA

The relevance of the in vitro human pDC experiments was explored in a murine splenocyte culture modeling immune responses to Ags (e.g., circulating) in the spleen. The spleen is a specialized tissue enriched with dendritic cells that are responsive to circulating microbial and endogenous danger signals (23–25). As with isolated human pDCs, TFAM enhanced the induction of type I IFN responses by the TLR9 agonist, and this effect was RAGE dependent. Furthermore, inhibitors of endosomal acidification (chloroquine), PI3K signaling (LY294002) and NF-κB signaling (BAY) attenuated type I IFN production in response to TFAM and CpGA DNA (Fig. 6B). Conversely, there was no significant effect upon Ag-induced IFN-α release following pretreatment with HMGB1 blocking Ab. As expected, enhanced (~2-fold) proliferation of pDCs in the spleen consequent to Flt3L expansion (Fig. 6A) was associated with an increased response to the TLR9 agonist (Fig. 6B). As in human pDCs, heparin pretreatment further supported the significance of RAGE involvement by demonstrating dose-dependent inhibition of TFAM plus CpGA DNA-induced IFN-α release (Fig. 6C). This was shown even more relevant by the significant reductions in type I IFN activation in splenocytes from RAGE<sup>−/−</sup> mice (Fig. 6D).

As expected, corresponding cell signaling experiments demonstrated that relative expressions of p-Akt, p-ERK and p-NF-κB were amplified within 15–30 min of CpGA DNA treatment alone or in combination with TFAM (Fig. 7). Pretreatment with inhibitors of PI3K (LY294002) and NF-κB (BAY) greatly attenuated the associated p-Akt (Fig. 7A, 7B) and p-NF-κB (Fig. 7C, 7D) expressions, as anticipated. PI3K inhibition markedly decreased p-ERK expression as well (Fig. 7B). Likewise, inhibition of RAGE
(sRAGE) notably diminished the expression of p-ERK but had no detectable effect upon p-Akt or p–NF-κB expressions (Fig. 7B, 7D).

**Discussion**

The cause of sterile inflammation in the context of cell necrosis (tissue damage) is a topic of great interest and ongoing controversy (1). Mitochondria are of particular interest given their bacterial origins and retention of bacterial features, including CpG-enriched DNA encoding for N-formylated peptides (2–6). Previous studies have documented the proinflammatory actions of mitochondrial DAMPs released into various body compartments, including blood (6), synovial fluid (2), and liver tissue (26); however, uncertainties exist relating to how mitochondrial Ags engage immune cells to promote inflammation.

pDCs play an essential role as sentinels of the immune system, providing immune surveillance for potentially hazardous exposures (7) and regulating the balance between innate and adaptive immune responses (27). pDCs are present in most tissues where they serve as excellent Ag presenters and are the primary producers of type I IFNs in response to activation of specialized receptors that are sensitive to microbial RNA (TLR7) and CpG motif-enriched DNA (TLR9) (7). pDCs also respond to endogenous danger signals (9). In the context of cell necrosis, the current study indicates that the mitochondrial fraction is by far the most potent in terms of activating pDCs and further incriminates TFAM and mitochondrial (nonmethylated CpG-enriched) DNA as mediators of this response.

HMGB1 is a nuclear protein that is believed to cause inflammation when released into the extracellular space, particularly following cell necrosis (15, 28). However, HMGB1 is a “sticky” protein that binds avidly to DNA and to bacterial lipids, including endotoxins (5, 29), and contamination with the latter likely contributes to the potent activation of inflammatory cells by recombinant HMGB1 produced in *E. coli* (28, 30). Although highly purified HMGB1 is capable of binding mitochondrial DNA (5), it normally associates with nuclear DNA to promote transcription and repair or mobilizes to the cytoplasm during cell stress (10). TFAM is a functional and morphological homolog of HMGB1 (11), sharing a highly conserved DNA-binding sequence that is common among HMG family proteins (31, 32). TFAM is normally confined to the mitochondrial compartment where it is shown to strongly associate with mitochondrial DNA (12). TFAM is essential for mitochondrial DNA transcription and attenuates mtDNA breakdown (12). This study is the first, to our knowledge, to show that TFAM remains bound to mtDNA following cell necrosis and could thereby promote the activation of readily available APCs, particularly TLR9-expressing pDCs.

In keeping with the previous study by Tian et al. relating to HMGB1 (9), we found that exposure to TFAM alone was insufficient to elicit pDC activation; whereas, TLR9-mediated immune responses to CpG DNA were significantly amplified by TFAM through a RAGE-dependent mechanism. We further showed that RAGE, TLR9, and TFAM colocalize within endosomes and that endosomal processing was essential for pDC activation under these conditions. Our findings are in keeping with previous studies showing that RAGE agonists likely promote Ag binding to TLR9 within endosomes, thereby enhancing pDC activation (9). Further support for RAGE-mediated activation of pDCs by CpG DNA and TFAM is provided by the heparin experiments, wherein increasing titrations of heparin were shown to suppress pDC activation. Heparin is a potent competitive inhibitor of RAGE–ligand interactions and associated inflammatory responses (33, 34). In this regard, HMG family proteins and CpG DNA independently bind with high affinity to RAGE (35). We suspect that the association of TFAM with immunogenic mitochondrial DNA enhances the affinity of the latter for RAGE.

The signaling mechanisms by which pDC type I IFN responses are regulated have been recently identified. A recent study by Guiducci et al. (21) indicates that PI3K-dependent nuclear translocation of transcription factor IRF-7 is essential for pDC type I IFN responses. Our data from human pDCs confirmed that PI3K is essential for IFN-α gene transcription and protein release in response to TLR9 activation; however, PI3K did not significantly influence nuclear IRF-7 translocation. Our findings implicate other mechanisms, including PI3K-dependent activation of ERK and NF-κB. PI3K may also influence IFN-α transcription through the regulation of histone deacetylase enzymes (36, 37), which would explain the dramatic suppression of all *IFN1α* subtype transcripts by PI3K inhibition despite the demonstrated nuclear presence of IRF-7.

These mechanisms were further investigated in cultured splenocyte preparations designed to model more complex immune environments encountered in vivo. The spleen is enriched with a mixed population of dendritic cells, including pDCs (24, 25), which are induced to proliferate in response to PdB1L. The murine, cultured splenocyte experiments largely mirrored the findings in purified human pDCs in that TFAM and CpG DNA were shown to promote type I IFN responses, which depended upon RAGE and TLR9 receptors and signaling pathways involving endosomes, PI3K, ERK, and NF-κB. Thus, these pathways may be reasonable targets to limit the sterile inflammatory response in vivo.

In summary, the mitochondrial fraction of the cell was demonstrated to be the most potent in terms of promoting pDC-mediated type I IFN responses in the setting of cell necrosis, which was dependent upon TLR9 and RAGE activation. Mito-

**FIGURE 8.** Schematic representation of pDC activation by necrotic cells. To summarize the findings presented in this paper, the data demonstrated that mitochondrial DNA (mtDNA) remained in association with TFAM upon release from necrotic cells. TFAM and mtDNA then associated with cytotoxic pDCs (24, 25), which are induced to proliferate in response to PdB1L. The murine, cultured splenocyte experiments largely mirrored the findings in purified human pDCs in that TFAM and CpG DNA were shown to promote type I IFN responses, which depended upon RAGE and TLR9 receptors and signaling pathways involving endosomes, PI3K, ERK, and NF-κB. Thus, these pathways may be reasonable targets to limit the sterile inflammatory response in vivo.
chondrial DNA, which is enriched in CpG motifs, and TFAM, a homolog of HMGB1, were incriminated as critical mitochondrial activators of TLR9 and RAGE, respectively. As shown schematically in Figure 8, our data further indicated that endosomal processing and PI3K, ERK and NF-κB signaling were essential for induction of type I IFN production by TFAM and CpGA DNA. Given that pDCs are potent regulators of innate and adaptive immune responses (27), and that mitochondrial DNA remains associated with TFAM after its release from damaged cells, this study identifies TFAM as a novel and potentially important endogenous danger signal.

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Disclosures

The authors have no financial conflicts of interest.

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