

1.5 Mitochondrial DNA Is a Pro-Inflammatory Damage-Associated Molecular Pattern Released During Active IBD

1.65

1.10

Ray K. Boyapati, MD,* David A. Dorward, PhD,* Arina Tamborska, BSc,* Rahul Kalla, PhD,*
Nicholas T. Ventham, PhD,* Mary K. Doherty, PhD,† Philip D. Whitfield, PhD,† Mohini Gray, PhD,*
Joseph Loane, FRCPATH,[§] Adriano G. Rossi, FBPhS,* Jack Satsangi, FRSE,† and Gwo-tzer Ho, FRCP*,†

1.70

1.15

Background: Due to common evolutionary origins, mitochondrial DNA (mtDNA) shares many similarities with immunogenic bacterial DNA. MtDNA is recognized as a pro-inflammatory damage-associated molecular pattern (DAMP) with a pathogenic role in several inflammatory diseases. We hypothesised that mtDNA is released during active disease, serving as a key pro-inflammatory factor in inflammatory bowel disease (IBD).

1.75

1.20

Methods: Between 2014 and 2015, we collected plasma separated within 2 hours of sampling from 97 prospectively recruited IBD patients (67 ulcerative colitis [UC] and 30 Crohn's disease [CD]) and 40 non-IBD controls. We measured circulating mtDNA using quantitative polymerase chain reaction (amplifying mitochondria *COXIII/ND2* genes) and also in mouse colitis induced by dextran sulfate-sodium (DSS). We used a mass spectrometry approach to detect free plasma mitochondrial formylated peptides. Furthermore, we examined for mitochondrial damage using electron microscopy (EM) and TLR9 expression, the target for mtDNA, in human intestinal IBD mucosa.

1.80

1.25

Results: Plasma mtDNA levels were increased in UC and CD (both $P < 0.0001$) compared with non-IBD controls. These levels were significantly correlated to blood (C-reactive protein, albumin, white cell count), clinical and endoscopic markers of severity, and disease activity. In active UC, we identified 5 mitochondrial formylated peptides (the most abundant being fIMMYALF with known chemoattractant function) in plasma. We observed mitochondrial damage in inflamed UC mucosa and significantly higher fecal MtDNA levels (vs non-IBD controls [$P < 0.0001$]), which supports gut mucosal mitochondrial DAMP release as the primary source. In parallel, plasma mtDNA levels increased during induction of acute DSS colitis and were associated with more severe colitis ($P < 0.05$). In active IBD, TLR9+ lamina propria inflammatory cells were significantly higher in UC and CD compared with controls ($P < 0.05$).

1.85

1.30

Conclusions: We present the first evidence to show that mtDNA is released during active IBD. MtDNA is a potential mechanistic biomarker, and our data point to mtDNA-TLR9 as a therapeutic target in IBD.

Key Words: mitochondrial DNA, DAMPs, TLR9

1.90

1.35

INTRODUCTION

The most widely accepted hypothesis underpinning the etiology of Crohn's disease (CD) and ulcerative colitis (UC) involves 4 pathogenic components: a dysregulated immune

1.95

1.40

Received for publications September 13, 2017; Editorial Decision January 26, 2018.

From the *MRC Centre for Inflammation Research, Queens Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom; †Gastrointestinal Unit, Western General Hospital, University of Edinburgh, Edinburgh, United Kingdom; ‡Centre for Health Science, University of the Highlands and Islands, Inverness, United Kingdom; §Department of Pathology, Western of General Hospital, Edinburgh, United Kingdom.

1.45

Conflicts of interest: The authors have no conflicts to declare.

Supported by: MRC grant G0701898, Crohn's and Colitis UK M16-1 to Gwo-tzer Ho; Edinburgh GI Trustees Grant (2014) to Ray Boyapati; Medical Research Society UK Vac-982-2016 to Arina Tamborska, and Wellcome Trust grant WT096497 to David Dorward and Adriano Rossi.

1.50

Address correspondence to: Gwo-Tzer Ho, FRCP, PhD (Edin.), MRC Centre for Inflammation Research, Queens Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, UK (gho@ed.ac.uk).

1.55

© 2018 Crohn's & Colitis Foundation. Published by Oxford University Press. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com.

1.57

doi: 10.1093/ibd/izy095

Published online XX XXXX 2018

response, genetic susceptibility, abnormal microbial composition, and environmental triggers.¹ There are clear differences between CD and UC. However, failure to resolve mucosal inflammation (which commonly reactivates upon withdrawal of anti-inflammatory treatments) is a notable shared clinical feature. In 1994, the "danger hypothesis" was proposed, in which immune responses are geared toward recognizing danger, irrespective of whether these signals arise endogenously or exogenously.² Endogenous damage-associated molecular patterns (DAMPs) are "danger signals" or "alarmins" released during host cellular stress or injury. Along with exogenous pattern-associated molecular patterns (PAMPs) of microbial origins, DAMPs can initiate and perpetuate an inflammatory response typically via germline encoded pattern recognition receptors (PRRs). We (and others) have recently suggested that DAMPs represent underexplored but potentially important pathogenic stimuli that maintain the state of abnormal mucosal inflammation in inflammatory bowel disease (IBD).³

1.100

1.105

1.110

We recently showed that gut mitochondrial dysfunction can result in loss of epithelial barrier function and the development of colitis.⁴ In this study, we focus on mitochondria as a source of DAMPs in IBD. Mitochondria are intracellular

1.114

double-membrane-bound organelles with many essential physiological roles such as energy production, regulation of cell death, and immune responses.⁵ Mitochondria are evolutionarily derived from energy-producing alpha bacteria, engulfed by archezoan cells approximately 2 billion years ago, leading to the symbiotic relationship that forms the basis of the eukaryotic cells.⁶ As such, mitochondria share several features with their bacterial ancestors, notably with a double-membrane structure and an independently replicating genome rich in hypomethylated CpG motifs, very similar to bacterial DNA. Just as the innate immune system recognizes conserved bacterial molecules, mitochondrial constituents are similarly immunogenic, acting as DAMPs during their uncontrolled release.⁷

The mitochondrial DAMP that has thus far attracted the most attention is mitochondrial DNA (mtDNA). Collins et al. first reported the inflammatory potential of mtDNA in 2004, when they found that mtDNA (and not nuclear DNA) induced tumor necrosis factor alpha (TNF α) and caused inflammatory arthritis when injected into the joints of the mice.⁸ MtDNA activates multiple pathways, notably toll-like receptor (TLR)–9, when it is released extracellularly, and through the cytosolic NLRP3-inflammasome and STING pathways.⁷ It is proposed that extracellular mtDNA mainly (but not exclusively) mediates its pro-inflammatory action via TLR9, which then proceeds through MyD88, triggering MAPK and NF κ B signaling,⁹ or through interferon regulatory factor 7 (IRF7) to enhance type 1 interferon response in dendritic (DC) or other immune cells. Uncontrolled mtDNA release is evident during conditions associated with acute tissue injury such as systemic inflammatory response syndrome (SIRS), fulminant liver failure, and sepsis,^{9–11} and in chronic inflammatory states such as systemic lupus erythematosus (SLE).¹² Given the significant tissue injury burden typically observed in active IBD, we hypothesized that such pathogenic release is present and that mtDNA can act as a pro-inflammatory DAMP potentiating and perpetuating the abnormal inflammatory response.

METHODS

IBD and Control Cohorts

Individuals were recruited from outpatient and inpatient settings from the Gastrointestinal Unit, Western General Hospital, Edinburgh, between April 2014 and November 2015. For the IBD cohort, recruited patients fulfilled the Lennard-Jones criteria of CD or UC.¹³ IBD patients were classified into CD or UC based on clinical, endoscopic, and histologic criteria. In addition, individuals with irritable bowel syndrome (IBS) or with no history of IBD and no gastrointestinal (GI) symptoms were recruited as non-IBD controls. IBS individuals had altered bowel habits and were defined following normal ileo-colonoscopy, stool calprotectin, and blood parameters. Individuals were excluded if they were younger than age 18 years or were unable to give written consent. Individuals with indeterminate

colitis and non-IBD colitis (eg, infective and microscopic) were excluded.

Biological material from subsets of prospectively recruited patients were used for:

1. fecal mtDNA analysis (n = 12 active UC vs 12 non-IBD controls);
2. mass spectrometry analysis (n = 5 acute severe UC vs 5 non-IBD controls);
3. electron microscopy (n = 6 active UC vs 6 non-IBD controls);
4. UC patients with longitudinal plasma sampling pre- and postcolectomy (n = 8).

All clinical and biological material/data acquisitions were carried out under Lothian Bioresource ethics approval 15/ES/0094.

Clinical Phenotype: Disease Activity

For UC and CD, disease activity was classified using the Simple Colitis Clinical Activity Index (SCCAI) and Harvey Bradshaw Index (HBI), respectively. Clinical remission was defined as an SCCAI of less than or equal to 2 for ambulatory ulcerative colitis patients, and an HBI of less than 5 for ambulatory patients with Crohn's disease. "Ambulatory" patients were defined as outpatients. Inpatients were further classified into acute severe disease if they required intravenous steroids for CD or fulfilling the Truelove & Witts criteria for UC. Endoscopic assessment of disease severity was obtained from endoscopic reports generated by GI physicians in charge of patients at the time of sample collection. Further clinical (stool frequency, temperature, and pulse) and disease severity characteristics, including laboratory assessments (full blood count, C-reactive protein, and serum albumin), were collected at the time of recruitment and blood sampling.

Human Plasma Processing

Venepuncture was performed by a trained clinician with a 21-gauge butterfly needle; 12–18 mL of blood including at least 9 mL in an ethylenediaminetetraacetic acid tube (Vacuette) was collected and processed within 2 hours. EDTA blood was centrifuged at 1000 g for 10 minutes at 4°C, and the plasma fraction was transferred to a 15-mL Falcon tube; this was then centrifuged at 5000 g for 10 minutes at 4°C to remove platelets and microparticles and achieve "cell free plasma." Plasma was divided into 0.5-mL aliquots and stored at –80°C until further use.

Extraction of mtDNA from Plasma

DNA was isolated using the QIAamp DNA Blood Mini Kit, per the manufacturer's instructions¹⁴; 200 μ L of plasma was used for each sample. Purity of the DNA was determined using Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and all DNA samples had OD260/OD280 values of 1.7–2.0.

Stool Processing

DNA was isolated using the QIAamp DNA Stool Mini Kit, per the manufacturer's instructions. To minimize cellular disruption (and thus minimize processing-related liberation of DAMPs), phosphate-buffered saline (PBS) was used instead of Buffer ASL in step 2 of the protocol.

Quantitative Polymerase Chain Reaction Protocols

Creation of standard curves

Standard curves for absolute quantification of circulating free mtDNA were developed using a modified protocol of that described previously.¹⁴ Mitochondria were extracted from cultured HepG2 cells using the Sigma Mitochondrial Isolation Kit for cultured cells using the manufacturer's instructions. Isolated mitochondria were stored at -20°C until further use. DNA was extracted using the QIAamp DNA Micro Kit (Qiagen, Valencia, CA, USA), per the manufacturer's instructions. Primer sequences (cytochrome C oxidase subunit III (*COXIII*): forward ATGACCCACCAATCACATGC, reverse ATCACATGGCTAGGCCGGAG; NADH-dehydrogenase 2 (*ND2*): forward CACAGAAGCTGCCATCAAGTA; reverse CCGGAGAGTATATTGTTGAAGAG) were blasted against the human genome and known bacteria to ensure selectivity for human mtDNA (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

mtDNA primer products were amplified by conventional polymerase chain reaction (PCR). PCR conditions were Stage 1: 95°C for 2 minutes; Stage 2: 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds; Stage 3: 72°C for 5 minutes. The PCR product was then run on a 4% agarose gel (2 g of agarose resuspended in 50 mL 1x TBE with 5 μL of Gel Red added to the solution once the agarose dissolved); 10 μL of PCR product was used, and samples were run next to a 100-bp DNA ladder. PCR product size was as expected (103 bp for *COXIII*, 90 bp for *ND2*). A QIAgen PCR purification kit was used in accordance with the manufacturer's instructions. The DNA was then eluted with 50 μL of Buffer EB into a sterile 1.5-mL Eppendorf and stored at -20°C until use. Isolated DNA was quantified by nanodrop (ThermoScientific). Standards were created from serial 10-fold dilutions of the PCR primer product.

Absolute quantification

Primers (MWG Eurofins) were suspended in 100- μM stock solution with DEPC-treated water and stored at -20°C before use. Subsequently, 20x primer solution (1.8 μM) was made (3.6 μL forward, 3.6 μL reverse, 192.8 μL DEPC- H_2O). In MicroAmp Optical 384-Well Reaction Plates (Applied Biosystems), 7 μL of master mix containing 5 μL 2x SYBR Green Fast mix (Applied Biosystems), 0.5 μL 20x primer mix, and 1.5 μL DEPC-treated water were mixed with 3 μL of

isolated DNA sample or standard. All reactions were carried out in duplicate, and discordant results were retested. All plates contained wells with no DNA as a negative, no-template control. Quantitative PCR (qPCR) reactions were conducted in an ABI7900 Fast Real-Time PCR System (Applied Biosystems) with the following settings: Stage 1: 95°C for 20 seconds; Stage 2: 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds; Melt curve: 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds, 60°C for 15 seconds. Absolute quantification of mtDNA was determined relative to the standard curve based on the following equation (as described by Chiu et al.¹⁴):

$$C = Q \times V_{\text{DNA}} / Q_{\text{PCR}} \times 1 / V_{\text{ext}}$$

- C target concentration in plasma or serum (copies per milliliter);
- Q target quantity (copies) determined by sequence detector in PCR;
- V_{DNA} total volume of DNA obtained after extraction, typically 50 μL per extraction;
- V_{PCR} volume of DNA solution used for PCR, typically 3 μL ;
- V_{ext} volume of plasma, typically 200 μL .

Amplification efficiency between 90% and 110% was taken as acceptable where slope refers to the gradient of the standard curve: (efficiency = $10^{(-1/\text{slope})} - 1$). The coefficient of determination value was also calculated with $r^2 > 0.985$. Melt curves were run to identify the presence of a primer dimer peak.

Mass Spectrometry

Plasma samples (100 μL) were acetone precipitated, dried down under vacuum, and reconstituted in 0.5% acetic acid. Peptides were then analyzed by Liquid chromatography tandem mass spectrometry (LC-MS/MS) in positive ion mode using a Thermo LTQ-Orbitrap XL mass spectrometer (Hemel Hempstead, UK) coupled to a Waters nanoAcquity UPLC system (Manchester, UK) with a linear gradient over 39 minutes (mobile phase A: 0.5% acetic acid in water; mobile phase B: 0.5% acetic acid in acetonitrile). N-formylated hexapeptides were identified on the basis of their accurate mass, retention times, and characteristic fragmentation patterns compared with custom synthesized standards (Peptide Protein Research Ltd, Fareham, UK). Quantification was achieved using a corresponding stable isotope labeled internal standard and calibration curve for each N-formylated hexapeptide.

Mouse Experiments and Induction of Colitis

C57/BL6 wild-type were used in experiments carried out under Home Office Project Licence PPL 70/8847 (G.T.H.). Acute colitis was induced by 2% DSS (MP Biomedicals Ltd) in drinking water ad libitum for 7 days. Mice were monitored daily for weight, presence of diarrhea, and blood. Mice were killed using CO_2 , and blood collection was carried out using direct cardiac puncture (~ 500 μL) into EDTA eppendorfs. Like human plasma processing, EDTA blood was centrifuged

at 1000 g for 10 minutes at 4°C, and the plasma fraction was transferred and further centrifuged at 5000 g for 10 minutes at 4°C to remove platelets and microparticles.

Transmission Electron Microscopy

Colonic pinch biopsies from IBD and non-IBD individuals were obtained from the distal colon during colonoscopy, briefly washed with sterile PBS, and immediately transferred into 3% EM grade glutaraldehyde solution in 0.1-M Sodium Cacodylate buffer, pH 7.3, for 2 hours before further processing (details available on request). For mouse studies, colons were flushed with PBS before transfer into EM solution, as above. All TEMs were carried out at the Electron Microscopy Unit, King's Building, University of Edinburgh.

Immunohistochemistry

Immunohistochemistry for anti-TLR9 (1 in 50 after Tri-EDTA antigen retrieval; Abcam ab52967) was performed on pseudo-anonymized human IBD and non-IBD colonic resection samples provided by the pathology department (J.L.) of the Scottish Tissue Bank via Scottish Academic Health Sciences Collaboration (SAHSC) SR493. All IBD samples were coded and matched (sex, age, and tissue location) with a non-IBD control group.

Statistical Analysis

Data are presented as numbers, percentages, means \pm standard error of the means (SEMs), and medians \pm interquartile ranges (IQRs) for parametric and nonparametric data, respectively. Student *t* and Mann-Whitney statistics were used for parametric and nonparametric data, respectively. Receiver operating characteristic (ROC) analyses were carried out using the following parameters: mtDNA, C-reactive protein, and albumin levels to predict the need for colectomy. Multivariate logistic regression was performed to assess variables predictive of high mtDNA. The Wilcoxon matched-pairs signed-rank test was used to determine the difference between matched pre- and postcolectomy mtDNA levels. Spearman's correlations were calculated to evaluate the relationship between mtDNA level and other biochemistry, and between *COXIII* and *ND2* qPCR results. Statistical analyses were performed using Graphpad, version 7 (Graphpad Software, San Diego, CA, USA), and SPSS, version 22 (IBM Corp., Chicago, IL, USA). Two-sided *P* values of <0.05 were considered statistically significant.

RESULTS

Increased Circulating Plasma mtDNA in UC and CD

We prospectively recruited and collected plasma from 97 IBD patients (67 UC and 30 CD patients) and 40 non-IBD

controls (20 healthy [HC] and 20 irritable bowel syndrome [IBS] controls) (Table 1). In all our samples, we performed qPCR using 2 sets of primers flanking *COXIII* and *ND2* genes of the mitochondrial genome. *COXIII* and *ND2* data were highly correlated ($r = 0.84$; $P < 0.0001$) (Supplementary Fig. 1), and *COXIII* data indicative of mtDNA release are presented herein. Overall, we found significantly higher levels of circulating cell-free plasma mtDNA in IBD (167.8 copies/ μ L; IQR, 78.06–387.2 copies/ μ L) compared with HC (64.6 copies/ μ L; IQR, 51.6–104 copies/ μ L; $P = 0.0002$) and IBS (44.6 copies/ μ L; IQR, 27.9–134.7 copies/ μ L; $P < 0.0001$). There was no difference between HC and IBS, and these groups were combined as non-IBD controls (Fig. 1A). Plasma mtDNA levels were significantly higher in both UC (172.3 copies/ μ L; IQR, 74.4–393.2 copies/ μ L; $P < 0.0001$) and CD (136.7 copies/ μ L; IQR, 88.0–370.9 copies/ μ L; $P < 0.0001$) compared with non-IBD controls (61.5 copies/ μ L; IQR, 32.8–104 copies/ μ L) (Fig. 1B).

In UC, individuals with acute severe disease, as defined by Truelove and Witts criteria and severe endoscopic appearances, have the highest mtDNA levels (clinical remission: 53.77 copies/ μ L; IQR, 30.56–86.8 copies/ μ L; vs severe UC: 234.7 copies/ μ L; IQR, 115.3–723.4 copies/ μ L; $P < 0.0001$; and endoscopically mild: 33.11 copies/ μ L; IQR, 28.71–44.9 copies/ μ L; vs severe UC: 255.4 copies/ μ L; IQR, 96.71–641.4 copies/ μ L; $P < 0.001$) (Fig. 2A and B). In the smaller cohort of CD, higher mtDNA levels were observed in those with severely active CD (159.1 copies/ μ L; IQR, 90.17–421 copies/ μ L) compared with those in remission (79.92 copies/ μ L; IQR, 30.94–145.9 copies/ μ L; $P = 0.04$) (Fig. 2C). In acute severe UC patients who were hospitalized, mtDNA levels on admission in patients who went on to require subsequent emergency colectomy were higher than those of patients who responded to medical therapy (colectomy: 302.5 copies/ μ L; IQR, 139–1553 copies/ μ L; vs no colectomy group: 165 copies/ μ L; IQR, 66.75–253 copies/ μ L; $P = 0.04$). Comparative ROC analysis of mtDNA, C-reactive protein, and albumin demonstrated areas under curve (AUCs) of 0.71, 0.76, and 0.82 in predicting colectomy ($P = 0.04$, 0.01, and 0.002), respectively (Supplementary Fig. 2). In CD, only 1 individual had surgery during recruitment; hence similar analyses were not performed. In our overall IBD cohort, we found that mtDNA levels were significantly correlated with the severe disease markers C-reactive protein ($r = 0.33$, $P < 0.0001$), albumin ($r = -0.32$, $P < 0.0001$), and white cell count ($r = 0.37$, $P < 0.0001$) (Supplementary Fig. 3). We identified a group of individuals with very high mtDNA levels ($n = 18$, >600 copies/ μ L) and investigated if there were unique phenotypic characteristics that defined the group. Multivariate analysis showed that C-reactive protein was independently associated with very high mtDNA levels ($P = 0.007$) (Supplementary Table 1). Collectively, our data show that circulating plasma mtDNA can be detected and is significantly increased in UC and CD; mtDNA levels were associated with disease activity and severity.

TABLE 1: Baseline Characteristics of IBD and Controls

	IBD		Controls	
	CD	UC	HC	IBS
No.	30	67	20	20
Age	37 (27–44)	36 (28–51)	36 (32–46)	33 (27–42)
M/F	17/13	44/23	10/10	13/7
Current smoker	26%	21%	15%	25%
Crohn's disease				
	Clinical Remission & Ambulatory	Clinically Active & Ambulatory	Hospitalized (IV Steroids)	
Hb, g/dl	145 (137–151)	156 (138–158)	130 (125–137)	
WCC, x10 ⁹ /L	7.4 (5.4–15.1)	10.9 (7.7–11.9)	8.75 (7.4–11.6)	
Platelets, x10 ⁹ /L	253 (218–295)	509 (300–414)	334 (279–378)	
C-reactive protein	3.5 (1–7.5)	5 (3.5–8)	26 (12–62)	
Albumin	38 (33–40)	38 (37–38)	29 (27–34)	
HBI	1 (0–2)	7 (6–9)	7 (4–14)	
Ulcerative colitis				
	Clinical Remission & Ambulatory	Clinically Active & Ambulatory	Hospitalized (IV Steroids)	
Hb, g/dl	136 (128–151)	131 (123–146)	114 (104–130)	
WCC, x10 ⁹ /L	6.3 (4.7–7.5)	7.7 (6.3–8.8)	11.3 (8.3–14.6)	
Platelets, x10 ⁹ /L	292 (252–303)	305 (256–335)	414 (288–501)	
C-reactive protein	2 (2–5)	3 (2–17)	21 (10–54)	
Albumin	39 (38–40)	38 (35–40)	30 (25–34)	
SCCAI	0 (0-0)	6 (4–8)	7 (4–10)	

Data are presented as median (±interquartile range).

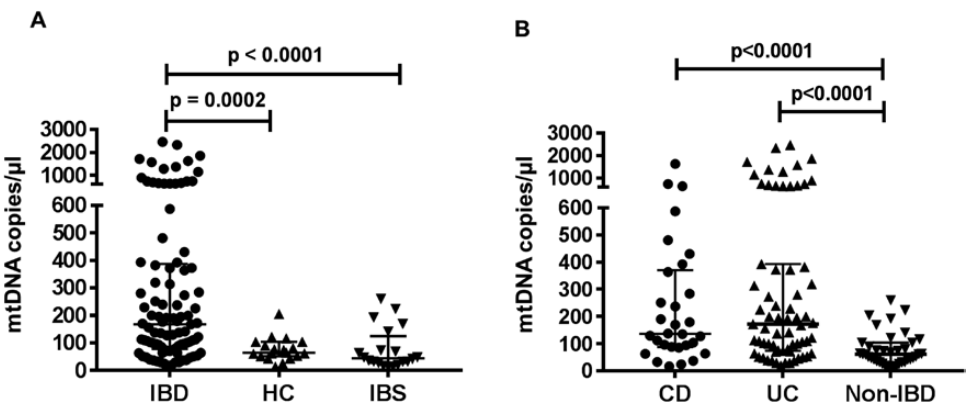


FIGURE 1. A, Plasma mtDNA (copy/μL) in IBD, HC, and IBS (n = 97, 20, and 20 samples respectively; $P \leq 0.001$). B, Plasma mtDNA (copy/μL) in CD, UC, and non-IBD (n = 30, 67, and 40 samples, respectively). Median ± interquartile range.

Mitochondrial N-Formylated Peptides are also Increased in Active IBD

A further shared feature of the mitochondria with bacteria is the production of short *N*-formylated peptides. Bacterial and mitochondrial proteins are the only source of

N-formylated peptides in nature. Mitochondrial *N*-formylated peptides are functionally similar to their bacterial counterparts, acting primarily as neutrophil chemoattractants.¹⁵ To provide further corroborative evidence, we employed a mass spectro-metric approach to identify and quantify *N*-mitochondrial

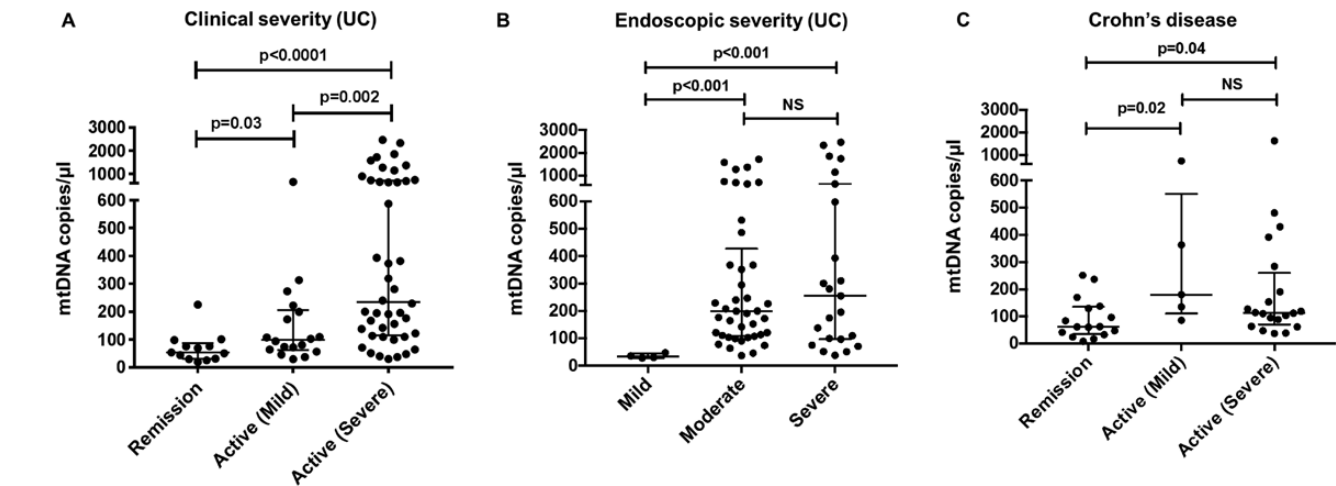


FIGURE 2. A, Plasma mtDNA (copy/μL) in UC in clinical remission (ambulatory), active (ambulatory), and severe active (hospitalized; n = 13, 18, and 44 samples, respectively; 8 UC individuals had samples taken at more than 1 time point during active disease and in remission). B, Categorized according to mild, moderate, and severe endoscopic appearances (n = 4, 41, and 23 samples, respectively). C, Plasma mtDNA (copy/μL) in CD, clinical remission (ambulatory), active (ambulatory), and severe disease (hospitalized; n = 10, 5, and 16 samples, respectively; 1 CD individual had samples taken at more than 1 time point during active disease and in remission).

formylated peptides in a subset of 5 acute severe UC vs 5 non-IBD controls. A screen for the free *N*-terminal hexapeptides of the 13 known mitochondrial encoded proteins confirmed the presence of 5 *N*-formylated termini (fMMYALF, fMTPMRK, fMNPLAQ, fMNFALI, and fMTMHTT) in acute severe UC samples. These were not detected in non-IBD controls. When quantified with synthetic standards, we found that the concentrations of each of these formylated peptides was significantly elevated ($P < 0.01$) in acute severe UC (Fig. 3). Of the mitochondrial *N*-formylated peptides, fMMYALF was the most abundant. This is highly relevant as fMMYALF is the most biologically active mitochondrial *N*-formylated peptide.¹⁶

Increased Plasma Mitochondrial DNA Release in Acute DSS Colitis

In order to provide further supportive data, we used the acute dextran sulfate sodium (DSS) colitis mouse model to test if plasma mtDNA can be detected as colitis develops. Similar to human IBD, we found significantly higher plasma mtDNA after acute DSS colitis (Fig. 4A), and high levels of mtDNA were associated with more severe colitis ($>10\%$ vs $<10\%$ weight loss at day 7 of DSS colitis; $P = 0.03$) (Fig. 4B). These findings in an acute colitis model supported our recent findings that also demonstrated increased plasma mtDNA in the chronic *mdr1a*-deficient colitis model.⁴ Hence, these data indicate that gut inflammation is associated with the release of mtDNA during active colitis.

Intestinal Mucosal Mitochondrial Damage and mtDNA Release in Active IBD

We recently showed that loss of a mitochondrial protective mechanism at the intestinal mucosal level rendered the

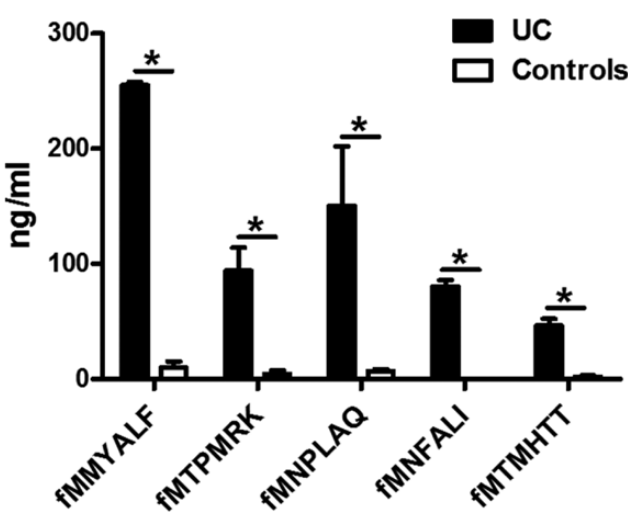


FIGURE 3. Mitochondrial formylated peptide quantification in 5 UC vs 5 non-IBD controls (* $P \leq 0.01$ for fMMYALF, fMTPMRK, fMNPLAQ, fMNFALI, and fMTMHTT). Mean \pm standard error of mean.

mitochondria susceptible to damage and triggered the onset of colitis in *multidrug resistant-1* (*mdr1*) deficient mice.⁴ Other relevant IBD mouse models with primary autophagy (*Irgm* and *Atg16l1*),^{17,18} including those with secondary autophagy impairments due to defective ER-stress¹⁹ and NLRP6 inflammasome activity,²⁰ all exhibited similar accumulations of damaged mitochondria within the gut epithelium as seen in *mdr1*-deficient mice. The findings of abnormal mitochondria have been demonstrated in human IBD.^{21–23} We confirmed these when we prospectively sampled pinch biopsies from affected colon tissue in individuals with active UC and non-IBD controls (Supplementary Table 2). Transmission EM of the colon showed

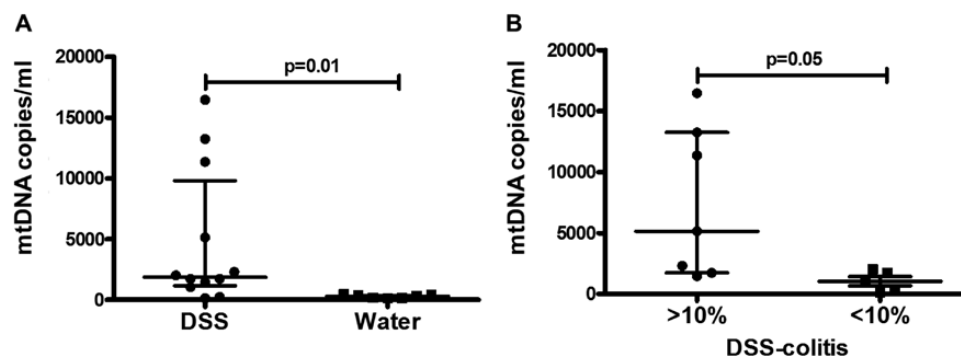


FIGURE 4. A, Plasma mtDNA (copies/mL) after acute DSS colitis vs controls (n = 12 and 14; $P = 0.01$).[†] B, Plasma mtDNA (copies/mL) stratified to < or >10% weight loss at day 7 of DSS colitis ($P = 0.03$). Median \pm interquartile range.

evidence of mitochondrial damage (with loss of inner cristae structure, increased lucency with swollen rounded appearances) in areas of cellular injury, and damaged mitochondria were also extravasated within the subepithelium in affected UC mucosa (n = 6/group; active UC vs non-IBD). (Fig. 5A; Supplementary Fig. 4A and B). The nature of EM did not allow more detailed discrimination of whether specific enterocyte or inflammatory cell types displayed a predilection toward mitochondrial damage. We hypothesized that mitochondrial DAMPs are released primarily from affected IBD mucosa. We prospectively sampled fecal samples from individuals with active severe UC and found significantly higher mtDNA levels ($P < 0.0001$) compared with non-IBD controls (Fig. 5B). Overall, fecal mtDNA levels were higher than plasma levels (~1000-fold), although this may be explained by the inherent nature of the different biological materials. In a longitudinal series of acute severe UC with paired samples pre- and postcolectomy (median $\Delta 107$ days; IQR, 89–189 days), plasma mtDNA fell to normal after colectomy (n = 8; $P = 0.008$) (Fig. 5C). Many lines of evidence suggest that mtDNA's DAMP effect is mediated via TLR9, which is expressed in many immune cells including dendritic cells, B cells, and the intestinal epithelium.²⁴ We analyzed TLR9 protein expression in human IBD colonic resection specimens and found increased frequency of lamina propria TLR9+ cells in active UC and CD (Fig. 6A and B). Collectively, these data suggest that extravasation of mtDNA primarily occurs at the inflamed mucosa level in active IBD, where TLR9 expression, its primary cellular target, is increased. Presently, the latter is associative, and more work is necessary to confirm the downstream effects of mtDNA on gut mucosal immune response. Taken together, these findings indicate that mtDNA-TLR9 signaling is an attractive therapeutic target and that mtDNA itself may have a role as a mechanistic biomarker.

DISCUSSION

We present data to show for the first time that significantly increased levels of mtDNA are found in active human IBD and in mouse colitis. Furthermore, plasma mtDNA levels correlate

with disease activity and severity. We corroborated our findings with a second known DAMP by demonstrating the presence of *N*-formylated peptides arising from the mitochondria in the plasma. In this context, we posit that increased mitochondrial damage occurs at the inflamed IBD mucosa (supported by human and mouse studies); mitochondrial DAMPs are released in a pathogenic manner (both quantitatively and qualitatively) where they can be detected in the circulation and in the stools. Given that many lines of evidence implicate their functional pro-inflammatory actions, these findings have direct translational importance.

MtDNA's role as a TLR9 agonist has received the most attention, whereas many inflammatory models show better outcomes when TLR9 signaling is abolished. *Tlr9*-deletion is protective against SIRS after systemic administration of mitochondrial DAMPs,²⁵ and in lung,²⁶ liver,²⁷ and kidney²⁸ injury models characterized by high mtDNA release. Blocking TLR9 using inhibitory ligands has been shown to improve mtDNA-driven mouse models of cardiac failure²⁹ and nonalcoholic steatohepatitis (NASH).³⁰ The role of mtDNA-TLR9 in IBD and intestinal inflammation, however, is more complex. TLR9 is expressed both in the epithelium and in resident and recruited lamina propria immune cells. Whereas NF κ B activation is pro-inflammatory,³¹ intestinal epithelial NF κ B activation is cytoprotective and important to maintaining barrier integrity.³² Hence, in *tlr9*-deficiency, the intestinal epithelium is postulated to have lower protective NF κ B activation and is more susceptible to injury. CpG oligonucleotide treatment in mouse studies using low-dose DSS in colitis showed either no difference or a protective role, with the beneficial effects seen before the induction of colitis.^{33–35} Furthermore, CpG oligonucleotide treatment given during colitis worsened inflammation.³⁶ A recent clinical study on TLR9 agonist in moderate to severe UC failed to show overall clinical improvement.³⁷ Hence, translating anti-TLR9 therapy in IBD is likely dependent on the stage of inflammation and the cellular context where TLR9 is blocked.

Our data show a promising future role for mtDNA (and DAMPs) as a mechanistic biomarker. We recently highlighted

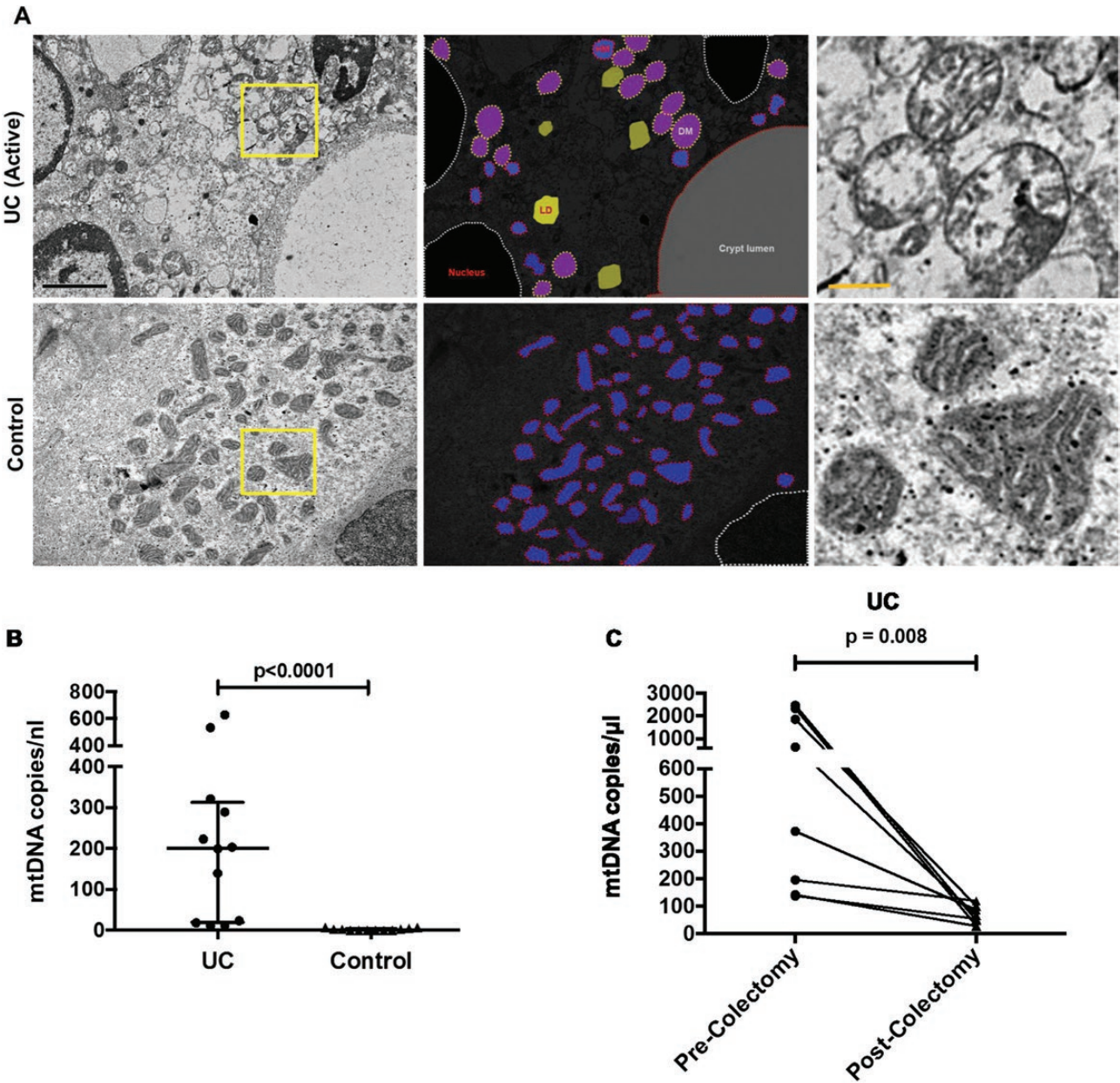


FIGURE 5. A, Representative transmission electron microscopy of distal colonic epithelium from active UC vs non-IBD controls (n = 6/group; bar = 5 μ m). Annotated image: purple indicates damaged mitochondria (DM), blue indicates healthy mitochondria (HM), and yellow indicates lipid droplets (LD). Black scale bar = 2 μ m. Yellow insert indicates damaged and healthy mitochondria from UC and controls, respectively (orange bar = 0.5 μ m). B, Fecal mtDNA (copy/nl) in active UC and non-IBD controls (n = 12/group). C, Longitudinal analysis of plasma mtDNA (copy/ μ L) in UC (n = 8 patients) during active disease and the same patient postcolectomy in clinical remission. All data represent median \pm interquartile range.

the importance of such biomarkers to identifying submechanisms that drive the heterogeneous clinical presentations and disease progression in IBD, where specific therapeutic interventions can be stratified accordingly. Clearly, more detailed studies are needed to fully test mtDNA's utility as a biomarker. There are limitations to our current data. First, we have not studied mtDNA in non-IBD intestinal inflammatory conditions such as infectious colitis or diverticulitis. It is conceivable

that high mtDNA release is also present. Second, our cohort was comprised of predominantly individuals with active UC (data for mitochondrial formylated peptides, EM, and fecal mtDNA were drawn from this group). It is not clear if similar findings are found in CD. It would be of interest to study a wider cohort of gut inflammatory conditions to determine whether mtDNA data are more specific to IBD or if mtDNA is a general marker for gut inflammation (like calprotectin).

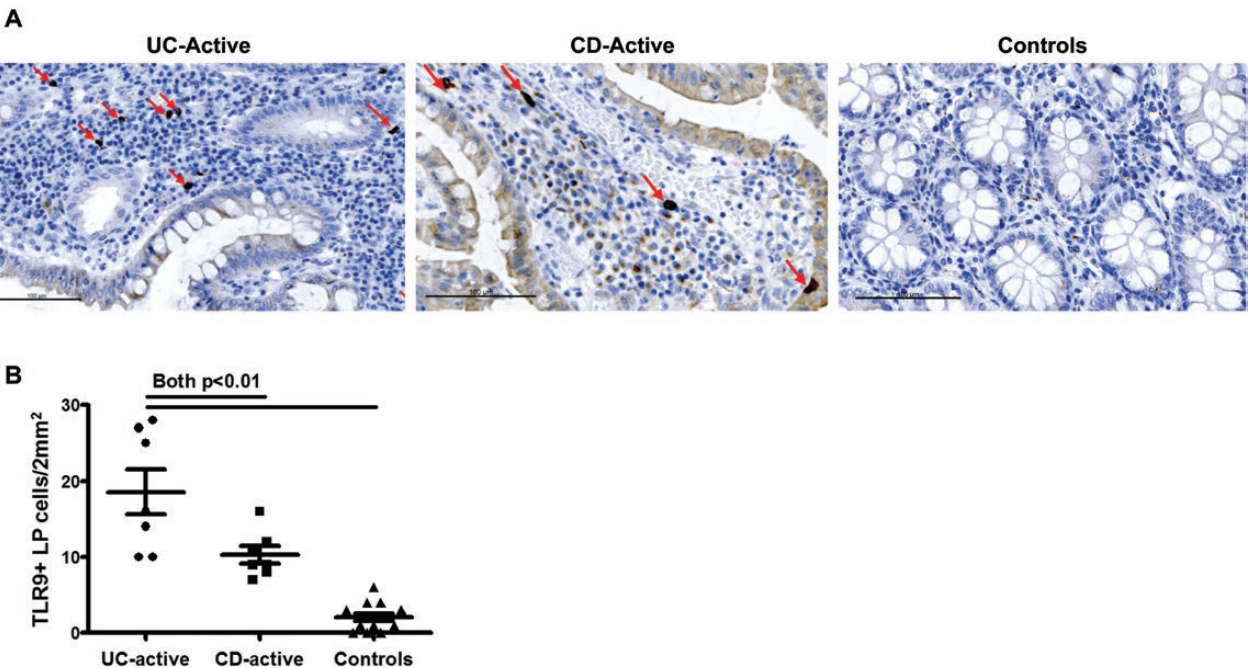


FIGURE 6. A, Immunohistochemistry for anti-TLR9 in human IBD colon (UC and CD: n = 7/group; vs non-IBD control: n = 14). Red arrows indicate TLR9-positive cells. Black scale bar = 100 μ m. B, TLR9+ cell counts in lamina propria (LP) of human IBD colon per 2 mm² (UC and CD: n = 7/group; vs non-IBD control: n = 14).^{*} Mean \pm standard error of mean.

There are many translational opportunities offered by targeting mitochondrial DAMPs. These include inhibiting mtDNA release (eg, diverting the manner of cellular death or active extrusion of mitochondrial DNA using pro-apoptotic caspases), reducing the inflammatory potential of mtDNA (eg, mitochondrial anti-oxidant treatment or DNAases to digest NET-bound mtDNA), augmenting damaged mitochondrial clearance mechanisms (eg, mitophagy activation), interfering with mtDNA-TLR9 activation (using inhibitory CpG ligands), and targeting downstream NF κ B and MAPK pathways in the relevant inflammatory cell groups (eg, neutrophils) (reviewed in³⁸). Similarly, much is known about the biological effects of *N*-formylated peptides and their cognate receptors (FPR1, -2, and -3). Activation of FPR1 drives neutrophil chemotaxis and stimulates a variety of antimicrobial responses, including degranulation, reactive oxygen species production, and cytokine release. We recently showed that both FPR1 gene deletion and pharmacologic inhibition are protective in inflammatory lung disease.^{6, 39, 40} There are other DAMPs such as calprotectin (s100a8/9), high mobility group box-1 (HMGB1), and interleukin (IL)-33 (reviewed in³) with known pro-inflammatory mechanisms found in active IBD. The relative importance of these DAMPs has not yet been clarified but, as it stands, offers a rich realm of further translational opportunities.

In conclusion, our study suggests that mtDNA acts as a DAMP promoting inflammation in IBD. More broadly, our findings open up a new mechanistic layer and further expand the current model of IBD pathogenesis to incorporate the

“danger” model. Hence DAMPs as “enemies within” represent a potential major player in addition to established data implicating genetic susceptibility and exogenous microbial and environmental factors in the pathogenesis of IBD.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

ACKNOWLEDGEMENTS

Author contributions: study concept and design: G.T.H., R.K.B., D.A.D.; acquisition of data: R.K.B., R.K., A.T., N.T.V., M.K.D., P.D.W.; analysis and interpretation of data: R.K.B., D.A.D., G.T.H.; drafting of the manuscript: R.K.B., D.A.D., A.G.R., J.S., G.T.H.; critical revision of the manuscript for important intellectual content: M.G., J.L., A.G.R., J.S., G.T.H.; technical and material support: M.G., J.L., M.K.D., and P.D.W.

REFERENCES

- Colombel JF, Mahadevan U. Inflammatory bowel disease 2017: innovations and changing paradigms. *Gastroenterology*. 2017;152:309–12.
- Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol*. 1994;12:991–1045.
- Boyapati RK, Rossi AG, Satsangi J, Ho GT. Gut mucosal DAMPs in IBD: from mechanisms to therapeutic implications. *Mucosal Immunol*. 2016;9:567–82.
- Ho GT, Aird RE, Liu B, et al. MDR1 deficiency impairs mitochondrial homeostasis and promotes intestinal inflammation. *Mucosal Immunol*. 2018;11:120–30.
- Friedman JR, Nunnari J. Mitochondrial form and function. *Nature*. 2014;505:335–43.

6. Vafai SB, Mootha VK. Mitochondrial disorders as windows into an ancient organelle. *Nature*. 2012;491:374–83.
7. West AP, Shadel GS. Mitochondrial DNA in innate immune responses and inflammatory pathology. *Nat Rev Immunol*. 2017;17:363–75.
8. Collins LV, Hajizadeh S, Holme E, et al. Endogenously oxidized mitochondrial DNA induces in vivo and in vitro inflammatory responses. *J Leukoc Biol*. 2004;75:995–1000.
9. Zhang Q, Raoof M, Chen Y, et al. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature*. 2010;464:104–7.
10. McGill MR, Kyung SY, Rogers AJ, et al. Circulating mitochondrial DNA in patients in the ICU as a marker of mortality: derivation and validation. *PLoS Med*. 2013;10:e1001577; discussion e1001577.
11. McGill MR, Staggs VS, Sharpe MR, et al; Acute Liver Failure Study Group. Serum mitochondrial biomarkers and damage-associated molecular patterns are higher in acetaminophen overdose patients with poor outcome. *Hepatology*. 2014;60:1336–45.
12. Caielli S, Athale S, Domic B, et al. Oxidized mitochondrial nucleoids released by neutrophils drive type I interferon production in human lupus. *J Exp Med*. 2016;213:697–713.
13. Lennard-Jones JE. Classification of inflammatory bowel disease. *Scand J Gastroenterol Suppl*. 1989;170:2–6; discussion 16.
14. Chiu RW, Chan LY, Lam NY, et al. Quantitative analysis of circulating mitochondrial DNA in plasma. *Clin Chem*. 2003;49:719–26.
15. Carp H. Mitochondrial N-formylmethionyl proteins as chemoattractants for neutrophils. *J Exp Med*. 1982;155:264–75.
16. Rabiet MJ, Huet E, Boulay F. Human mitochondria-derived N-formylated peptides are novel agonists equally active on FPR and FPRL1, while listeria monocytogenes-derived peptides preferentially activate FPR. *Eur J Immunol*. 2005;35:2486–95.
17. Adolph TE, Tomczak MF, Niederreiter L, et al. Paneth cells as a site of origin for intestinal inflammation. *Nature*. 2013;503:272–6.
18. Liu B, Gulati AS, Cantillana V, et al. Irgm1-deficient mice exhibit paneth cell abnormalities and increased susceptibility to acute intestinal inflammation. *Am J Physiol Gastrointest Liver Physiol*. 2013;305:G573–84.
19. Kaser A, Lee AH, Franke A, et al. XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. *Cell*. 2008;134:743–56.
20. Elinav E, Strowig T, Kau AL, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell*. 2011;145:745–57.
21. Delpre G, Avidor I, Steinherz R, et al. Ultrastructural abnormalities in endoscopically and histologically normal and involved colon in ulcerative colitis. *Am J Gastroenterol*. 1989;84:1038–46.
22. Söderholm JD, Olaison G, Peterson KH, et al. Augmented increase in tight junction permeability by luminal stimuli in the non-inflamed ileum of Crohn's disease. *Gut*. 2002;50:307–13.
23. Nazli A, Yang PC, Jury J, et al. Epithelia under metabolic stress perceive commensal bacteria as a threat. *Am J Pathol*. 2004;164:947–57.
24. Pedersen G, Andresen L, Matthiessen MW, et al. Expression of toll-like receptor 9 and response to bacterial CPG oligodeoxynucleotides in human intestinal epithelium. *Clin Exp Immunol*. 2005;141:298–306.
25. Tsuji N, Tsuji T, Ohashi N, et al. Role of mitochondrial DNA in septic AKI via toll-like receptor 9. *J Am Soc Nephrol*. 2016;27:2009–20.
26. Wei X, Shao B, He Z, et al. Cationic nanocarriers induce cell necrosis through impairment of Na⁺/K⁺-ATPase and cause subsequent inflammatory response. *Cell Res*. 2015;25:237–53.
27. Marques PE, Amaral SS, Pires DA, et al. Chemokines and mitochondrial products activate neutrophils to amplify organ injury during mouse acute liver failure. *Hepatology*. 2012;56:1971–82.
28. Bakker PJ, Scantlebury AM, Butter LM, et al. TLR9 mediates remote liver injury following severe renal ischemia reperfusion. *PLoS One*. 2015;10:e0137511.
29. Oka T, Hikoso S, Yamaguchi O, et al. Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure. *Nature*. 2012;485:251–5.
30. Garcia-Martinez I, Santoro N, Chen Y, et al. Hepatocyte mitochondrial DNA drives nonalcoholic steatohepatitis by activation of TLR9. *J Clin Invest*. 2016;126:859–64.
31. Zhang Q, Lenardo MJ, Baltimore D. 30 years of NF- κ B: a blossoming of relevance to human pathobiology. *Cell*. 2017;168:37–57.
32. Nenci A, Becker C, Wullaert A, et al. Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature*. 2007;446:557–61.
33. Rachmilewitz D, Katakura K, Karmeli F, et al. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology*. 2004;126:520–8.
34. Lee J, Mo JH, Katakura K, et al. Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nat Cell Biol*. 2006;8:1327–36.
35. Rose WA II, Sakamoto K, Leifer CA. TLR9 is important for protection against intestinal damage and for intestinal repair. *Sci Rep*. 2012;2:574.
36. Obermeier F, Dunger N, Strauch UG, et al. Contrasting activity of cytosin-guanosin dinucleotide oligonucleotides in mice with experimental colitis. *Clin Exp Immunol*. 2003;134:217–24.
37. Atreya R, Bloom S, Scaldaferrri F, et al. Clinical effects of a topically applied toll-like receptor 9 agonist in active moderate-to-severe ulcerative colitis. *J Crohns Colitis*. 2016;10:1294–302.
38. Boyapati RK, Tamborska A, Dorward DA, Ho GT. Advances in the understanding of mitochondrial DNA as a pathogenic factor in inflammatory diseases. *Fl000Res*. 2017;6:169.
39. Dorward DA, Lucas CD, Chapman GB, et al. The role of formylated peptides and formyl peptide receptor 1 in governing neutrophil function during acute inflammation. *Am J Pathol*. 2015;185:1172–84.
40. Dorward DA, Lucas CD, Doherty MK, et al. Novel role for endogenous mitochondrial formylated peptide-driven formyl peptide receptor 1 signalling in acute respiratory distress syndrome. *Thorax*. 2017;72:928–36.