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TITLE: Mitochondrial Debris as a Discriminator Between Inflammatory and Infectious Complications of Blast Injuries: The Enemy Within

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14. ABSTRACT

Background: Blast injuries kill tissue. The inflammatory response that follows is hard to tell from infection, but should be treated very differently. Objective: The objective is to create tests differentiating inflammation from infection. Rationale: Inflammation can be a response to infection or to the release of molecules that are normally from inside cells. Mitochondria are cellular structures that are evolutionarily derived from bacteria. Thus the body acts infected when they are released from damaged cells. We can tell mitochondrial from bacterial DNA using genetic tests. Specific aims: 1) Determine which patients have bacterial DNA and mitochondrial DNA in their blood by created tests measuring mitochondrial and bacterial DNA in rat plasma. We will start with screening tests, develop simpler tests that can be used in the battlefield. 2) Check the accuracy of our tests: We will create different injury and infection models to improve the tests. We will make sure tests that work in animals also apply to humans. 3) Determine if mitochondrial DNA is actually harmful. Mitochondrial DNA might be harmful or simply a marker for crushed cells. If it is harmful, we can block its effects. So we will test whether it worsens injuries of the lung in rats.

15. SUBJECT TERMS
mitrochondrial DNA, inflammation, bacteria, injuries

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INTRODUCTION

Blast injuries cause widespread tissues necrosis and this can result in a sterile Systemic Inflammatory Response Syndrome (SIRS) that closely mimics clinical sepsis. Since SIRS and sepsis are treated very differently, discrimination between host responses to blast injury and infection is critical for blast-injured soldiers. No current test modality has been shown to be capable of distinguishing Sepsis from SIRS. Thus our research plan was to close this knowledge gap using mitochondrial debris and universal bacterial DNA sequences as discriminators between the inflammatory and infectious complications of blast injuries. In the second year of our project we achieved the animal and human research objectives defined in the revised Statement of Work. The overall project has been very productive.

BODY

Our specific Aims were to:

1) Determine which patients have bacterial DNA and mitochondrial DNA in their blood

2) Check the accuracy of our tests and

3) Determine if mitochondrial DNA is harmful.

We have mad major progress on all of these fronts.

YEAR 1

We began by assessing the release of mtDNA and bDNA in human injuries relevant to battle conditions. Our first work relevant to the award was to complete validation of PCR primers for the identification of mtDNA and bDNA. This work also addressed technical concerns related to Aim 2 here (discussed more below). This work contributed to the publication of our seminal paper in *Nature*[1] (**Appendix 1**). This publication has now 'changed the playing field' for how inflammatory illnesses are understood. Confirmations of the principles we discovered have now been reported by multiple other groups [see McDonald et al, Science. 2010 Oct 15;330 (6002):362-6 and many others].

We then went on to evaluate the role of fracture injuries in mobilizing mtDNA from human tissue trauma. As proposed, we used discarded human samples to show that long bone fractures (very common in combatants) and their repair by clinical reamed nailing operations mobilized huge amounts of mtDNA from the fracture site into the systemic circulation. Moreover, we showed that a steep gradient exists from the fracture (and fracture repair sites to the bloodstream. This strongly implicates blunt tissue injuries (both in civilian and military trauma) as initiating or exacerbating systemic inflammation through the release of mitochondrial debris (MTD) generated at the site. These data are now published in the *Journal of Orthopedic Trauma*[2] **(Appendix 2)** and Journal of Trauma[3] (**Appendix 3**). The importance of formyl peptides derived from blunt tissue injury was also investigated in advanced studies of signal pathways leading to neutrophil chemotaxis[4].

We also proposed in Aim 1 to start creating screening tools for clinical use in austere environments such as forward deployments. In this regard we have 'fine-tuned' our PCR-based approach to diagnosis by constantly improving upon our primers (See Sursal et al **Appendix 4**). Our current PCRs now detect $<10^{12}$ g/mL of bacterial DNA from the major common early wound pathogens while failing to detect mtDNA at essentially any concentration. Thus we have succeeded in increasing the sensitivity and specificity of our assays as planned and at this point our assays detect femtogram /mL amounts of DNA both for mitochondrial (Cyto B) DNA and for bacterial 16s-rRNA in a

tube assay with undetectable cross reactivity. This achieves much of the thrust of Aim 2. A preliminary patent has been granted for this method.

During that funding period, we began collaborations with a bioengineering group at Boston University to create a 'PCR on a chip' approach aimed at use in austere-environment applications. This collaboration led to a CIMIT grant applies the approach and primers developed here to that PCR platform. We are well along in using their 'chip' platform. This also is not yet published due to patenting considerations, but our preliminary findings are appended (**Appendix 5**).

Our third goal was to de termine whether mitochondrial DAMPs were intrinsica lly harmful or if they were simply markers for cellular damage. In year 1 we determined that they are ***both***. Our work both in human PMN [1] and in human fe mur fracture repair models[2] showed that mitochondrial peptide s contribute to the neutrophil (PMN) activation that occurs after injury. There we showed that this activation occurs via P42/44 and that it leads to the activation of cellula r synthesis of the matrix metalloproteinase MMP-9. To examine the role of mtDNA as a discriminator of tissue trauma from tissue in jury by sepsis we be gan to co llaborate with another gr oup of researchers at BU. This group has been involved in sepsis resear ch using primate models (*papio*). They became interested in our work vi a our *Nature* publication and sought to u se our assays as bio markers for tissue injury in a primate model of *B. Anthracis* (Anthrax) sepsis. Although we have not created tra uma models in the baboon, we believe that some of the control conditions used in the Anthrax studies are excellent animal models of sterile cellular injury as proposed under th e revised statement of work. Under our direction the se collaborators have isolated mtDNA and bDNA from archived septic baboon plasma at their Level 3 bio-saf ety facility. We have analyzed those samples using novel PCR primers that we developed specifically for baboons. The results are remarkable for their clarity and obvious importance and they have now been accepted for publication in SHOCK **(Appendix 4).** We f ound that a dministration of Shiga-toxin (a purified bacterial toxin that is free from bacteria) causes no increase in the circulation of bDNA but causes release of mtDNA into the circulation (indicating cell injury). We also saw th at the mtDNA 'injury sig nal' fell to b aseline as injury resolves in this non-lethal model. Thus our meth ods detect 'tissue injury signals' from en venomation and discriminate them f rom bacterial sepsis events. We also saw that baboons given sublethal challenges of *E. coli* show evidence of concurrent sepsis and septic t issue injury using bDNA and mtDNA as biomarkers. Thus the degree of cellular injury is related to the bacterial titer in sepsis and the injury abates as sepsis resolves.

In these st udies we a lso assayed plasma bDNA and mtDNA after anthrax exposure. Our 16s-rDNA bacterial primers identif ied B. anthra cis DNA in the bloodstream as readily as *E. coli*. In these experiments activated Protein C (aPC, 'Xigris') was also used as a b iologic response modifier to mitigat e inflammatory responses to Anthrax. We saw tha t animals given aPC survived where animals g iven placebo died. The use of aPC had no effect on the rapid appearance of anthrax bDNA in the circulation afte r inoculation or on the slow time course o f its cleara nce documented by our b DNA primers. This is as predicted. On the othe r hand, we have also been able to show using our mtDNA pri mers that anthrax [which causes necrotizing infection of the mediastinum] generates the release of massive amounts of mtDNA into the circulation. In this case we saw that use of aPC markedly attenuated release o f mtDNA. Also, lesser or greater tissue "injury signals" as seen in untreated Anthrax compared with aPC-treated anthrax or compared with *E. coli* sepsis may be associated with survival. If the mtDNA signal can thus be confirmed as a prognostic marker in either significant sterile injuries or sepsis it would become enormously useful as a predictor of medical resource or ICU utilization. In the milit ary setting it might predict need for early

evacuation. Specific necrotizing infections such as Anthrax or the more common necrotizing soft-tissue infections might be distinguishable from other more common infections on the basis of the presence and titer of mtDNA, which we hope will be available in the austere environment using technology that will provide results within about 3 hours.

YEAR 2

In the first year we developed tests for mitochondrial DNA (mtDNA) and bacterial DNA (bDNA) in rat plasma, baboon plasma and human plasma. We began human, rat and baboon studies of the role of mtDNA and bDNA in the response to military injury. In the 2nd year we performed a wide variety of studies aimed at the revised statement of work. We began with further general, background work.

- We refined PCR based tests for mtDNA and bDNA to yield results in absolute amounts by creating reliable standards.
- We created and validated complex animal models of injury-specific release of mtDNA and bDNA and their effects upon immunity. These models include a chest concussion model that is combined with sepsis (in the form of cecal ligation and puncture (CLP), and SIRS (generated by injection of mitochondria). These have now been used to investigate the role of mitochondrial debris in local tissue injury and in the ability of the organism to mobilize innate immune cells to sites of injury. Moreover, we have uncovered a major new pathway linking injury to infection.
- We have done a large collaborative study of mtDNA and bDNA release in human trauma victims with injuries relevant to battle conditions using repository discard plasma samples.
- We have completed the critical study of the role of mtDNA in the prolongation of sepsis and SIRS that we had partially reported in the last report and defined the roles of mtDNA and bDNA in organ failure following anthrax sepsis by analysis of archived samples from septic baboons. These studies are ***in press*** at SHOCK.

We have used these basic advances to move on to the specific aims as below:

1) **The role of systemic sepsis and mitochondrial debris in the response to thoracic blast injury.**

A rat model was created using a thoracic blast that creates reproducible pulmonary contusion. After standardization of a severe but surviveable model, we then coupled that model to a) sepsis in the form of a) cecal ligation and puncture (CLP) and b) SIRS generated by intra-peritoneal injection of rat mitochondria. This latter model used rat mitochondria equivalent to that in 5% of the rat's liver, thus simulating 5% hepatic necrosis from a liver injury. We validated that plasma mtDNA was equivalent in rats that had a like weight of crushed liver placed in the peritoneum or injected with mitochondria (MT). Remarkably, in all ways, MT acted essentially identically to CLP, so we will show only the MT data here. We examined PMN trafficking from the blood to lung and peritoneum in response to chemokine (MIP and CINC) and monokine (IL-1β) gradients. All cytokine levels were quantified in absolute amounts by indexing to blood urea nitrogen (BUN), which equilibrates across all body fluids except urine. This allowed us to create pictures of absolute cytokine gradients across blood and tissues.

We found that thoracic blast produced a huge influx of neutrophils (PMN) into the lung bronchoalveolar lavage fluid (BALF). This influx was markedly attenuated by the presence of MT in the peritoneal cavity **(Figure 1)**. The neutrophils were essentially

diverted into the peritoneal cavity **(Figure 2)** by both the chemo-attractant effects of the MT. These include both direct chemoattraction[1] and marked secondary production of CXC chemokines **(Figure 3)**. Pulmonary alveolar permeability (as measured by albumen leak) was increased by PC but similarly decreased by intra-peritoneal inflammation, whether produced by CLP or sterile MT **(Figure 4)**. IL-1β was increased in the BALF by PC, and in the peritoneum by MT or CLP (not shown), indicating monocyte activation. Again, lung IL-1β increases after PC were decreased by peritoneal inflammation, whether sterile or infective. We interpret these events as indicating that distant injuries do not compound the effects of pulmonary blast injury on lung function. In fact (remarkably) they likely ***attenuate*** the effects of blast injury. However, the presence of sterile systemic injury and the release of MT (and likely other DAMPs) markedly decreases the trafficking of PMN to the lung due to diminished chemo-attractant production in the lung. This infers that ***the presence of systemic inflammation due to diffuse tissue blast injury and release of mitochondrial DAMPs will make the lung susceptible to infection by otherwise sub-infective bacterial innocula.*** We believe that we have uncovered a basic process in the response to wounding here that will be the basis of intense subsequent study and lead to improved outcomes for the blast-injured combatant.

These data are currently being refined and analyzed, but the major thrust of the work with mitochondria has been incorporated in an abstract that has already been accepted for plenary presentation at the winter meeting of the Eastern Association for the Surgery of Trauma (EAST) **(Appendix 6)** and will therefore be published rapidly in the Journal of Trauma. The work examining the relationship of infection (CLP) to pulmonary PMN trafficking will be submitted elsewhere.

2) Studies of mtDNA and bDNA release in human trauma.

We have now assessed the release of mtDNA and bacterial DNA (bDNA) in archived plasmas from a large cohort of trauma patients stratified by ISS. Because these patients were meant to include 'all comers' they include blunt and penetrating injuries as well as open wounds and gut injuries where we expect direct bacterial contamination as well as tissue injury. Samples from the prospectively collected repository were assayed using our newly synthesized primers but we did not yet have highly purified standards. Thus we were not able to report absolute concentrations, as we are now able. That said, we can now report important new data concerning the release of inflammatory DNAs into plasma in trauma. First we have confirmed in a large cohort of unselected patients that mtDNA (Cytochrome B) is released into the plasma after injury. The increase is of the order of 256-fold **(Figure 5).** This is less than we reported in *Zhang et al*, but still very substantial considering the wide spectrum of trauma patients. Interestingly, unlike our prior findings, in this cohort we do now see bacterial (16S) DNA in the blood soon after trauma **(Figure 6)**. The amounts appear to be much smaller using semi-quantitative methods, and this must be studied further using our newer methods that are fully quantitative. Moreover, since many of these patients had contaminated injuries it is logical that there may be bDNA present. We also found that there was a strong association between shock (as assessed by base deficit) and mtDNA **(Figure 7)**. There was also a robust association between mtDNA in the plasma and injury severity and later development of Acute Lung Injury **(Figure 8)**. Other purported DAMPs (HMGB1 and Histones) show no such associations. These studies fulfill all of the objectives of Specific Aim 1 and are currently under submission.

3) Studies on the pathogenetic mechanisms of MT and mtDNA

Our third major goal was to determine if mtDNA is harmful. Along with the animal experiments listed above, we planned to address this primarily by assessing the effects of mtDNA on endothelial permeability. We have accomplished this by the use of our novel model systems that rely on various different endothelial phenotypes (PMECs, PAECs, HUVECs etc) to assess the interactions of PMN and endothelial cells in the presence of various stimuli. This modeling system studies the ultimate end organ effects of SIRS in a very realistic and important way. We have found using this system that mtDNA and MT peptides are each important stimuli of PMN mediated changes in endothelial permeability. In **Figure 9**, we see that mtDNA is capable of stimulating $2x10^5$ PMN seeded on endothelial monolayers (AE cells here) to induce marked increases in EC permeability where neither mtDNA alone nor PMN without mtDNA can induce permeability. Moreover, the induction of permeability is reversed by oligodeoxynucletide (ODN TTAGGG, 5 μM) specific for mtDNA. This robust study has also identified that endothelial cells stimulated by mtDNA demonstrate activation of P38 MAPKinase **(Figure 10)**, P44/42 MAP Kinase (not shown) and thus activated, synthesize HMGB1, a potent inflammatory mediator. **(Figure 11)**. The cross-talk between the activated PMN and EC appears to be the result of marked up-regulation of endothelial adhesion molecules ICAM-1 and E-selectin **(Figure 12)** in the presence of mtDNA. This results in markedly increased adherence (not shown). Moreover, we have shown for the first time that mtDNA is taken up directly into pulmonary endothelial cells and localize to endosomes, where they can interact with endosomal TLR detectors for nucleic acid ***Danger*** signals **(Figure 13)**. These data are in revision at PLOS-One. Thus both our human studies and our *in vitro* studies have now confirmed our prior work and bring strong evidence to bear on our original specific aim of demonstrating that mtDNA is pathogenic as well as a marker for injury. They also establish important new mechanistic insights into the pathogenesis of ***Danger*** mediated inflammation that will be of primary importance in future management of the blast-injured combatant who is manifesting SIRS due to tissue destruction.

Fig. 1

PMN in BALF

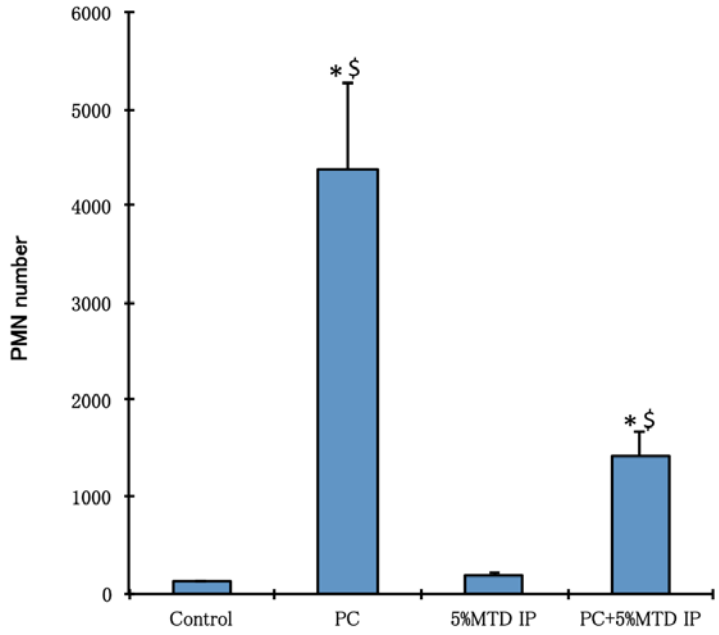


Fig. 1 (A). Pulmonary contusion (PC) causes marked PMN influx into the lung. Intraperitoneal (IP) 5%MT has no effect on PMN in the BALF but significantly decreases PMN trafficking to the lung by PC (n=8-10 rats/condition, * P< 0. 05 versus control, $ P< 0.05 PC versus PC+5%MT IP, ANOVA/ Dunn).

Fig. 2

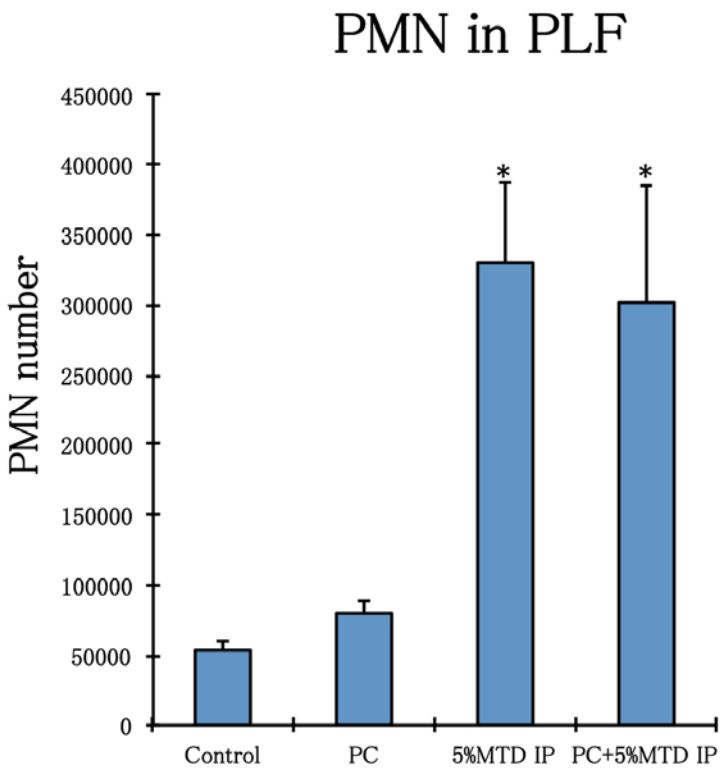


Fig. 2. **MT cause PMN to enter the peritoneal cavity.** Rats getting IP 5%MT or 5%MT+PC had marked peritoneal neutrophilia compared with control animals or animals with PC only (n=8-10/condition, * P < 0. 05 versus control or PC, ANOVA/ Dunn).

Fig. 3

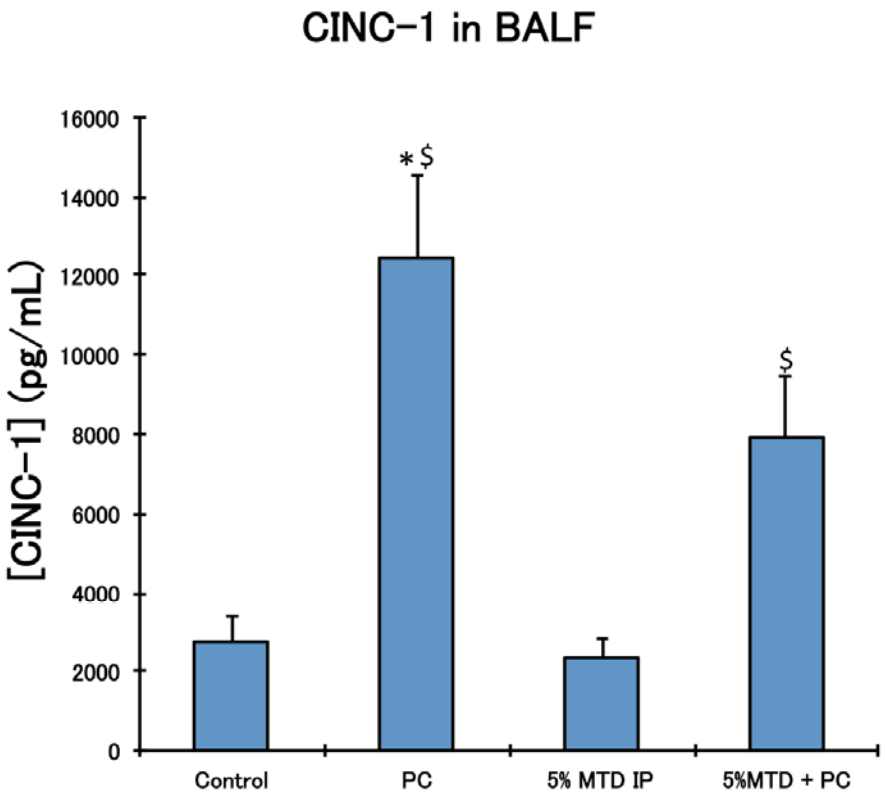


Fig. 3. Pulmonary contusion increases the chemoattractant CINC-1 in BALF significantly (n=7-9, * P < 0. 05 versus control, ANOVA/ Dunn's). The introduction of 5%MT into the peritoneum depresses this effect.

Fig. 4

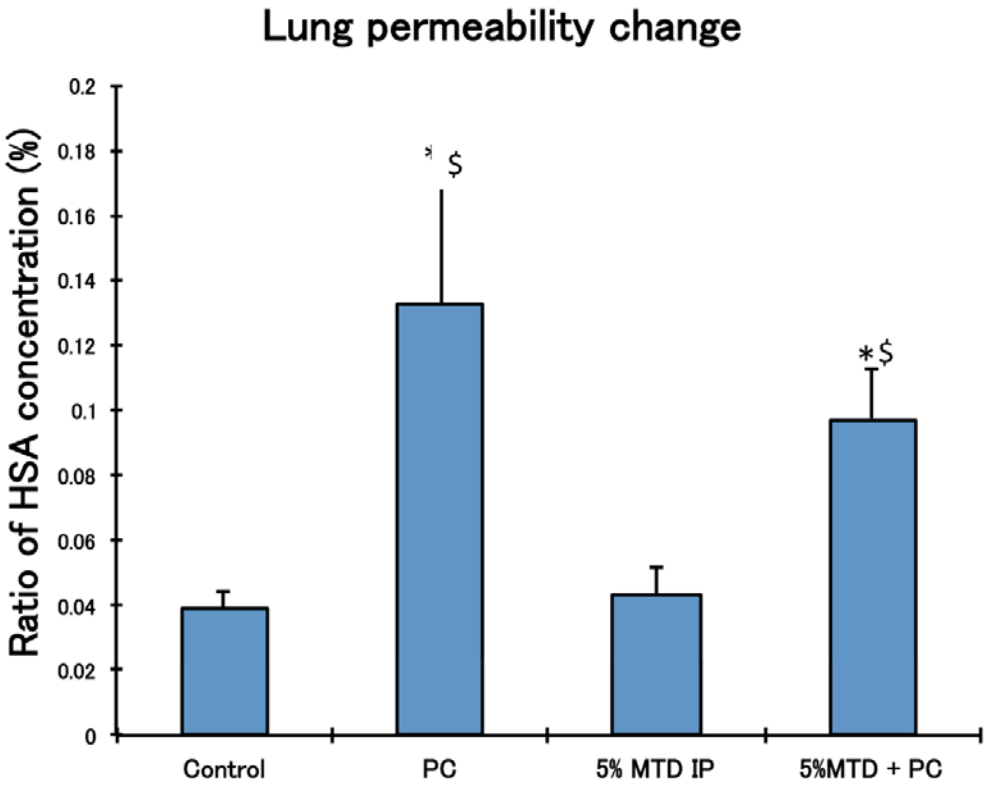
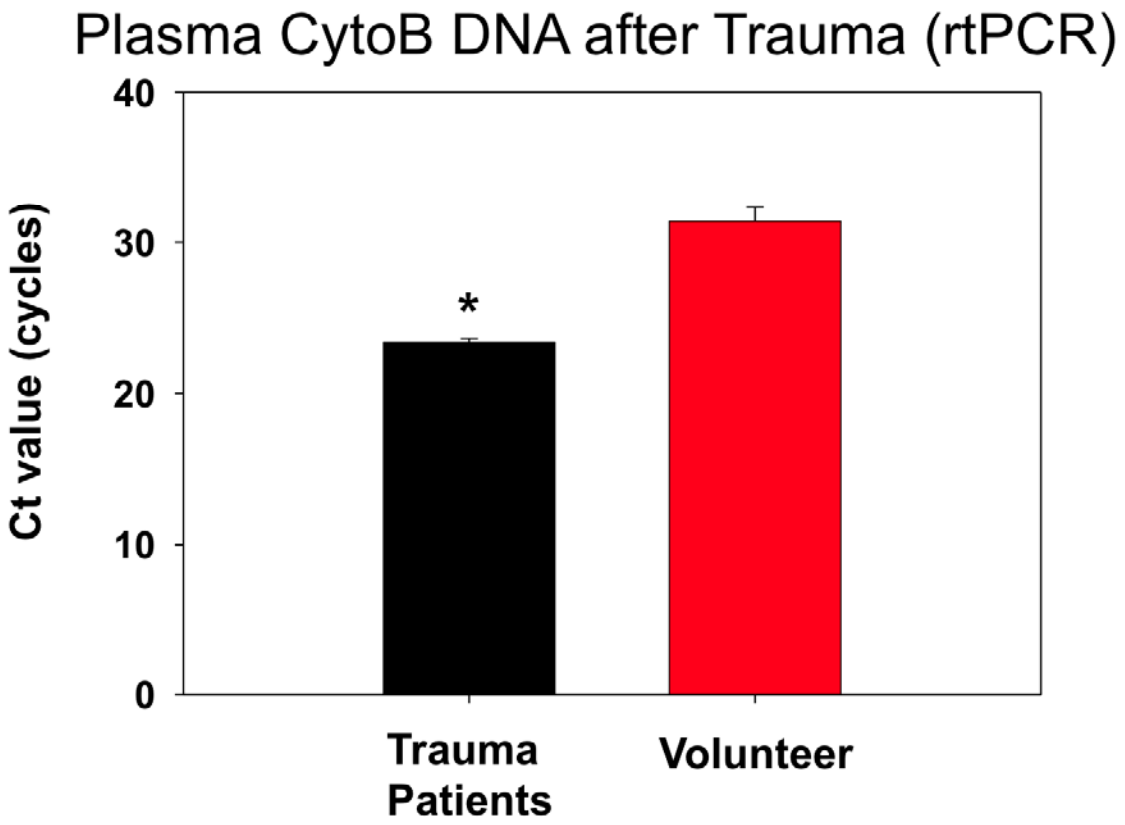


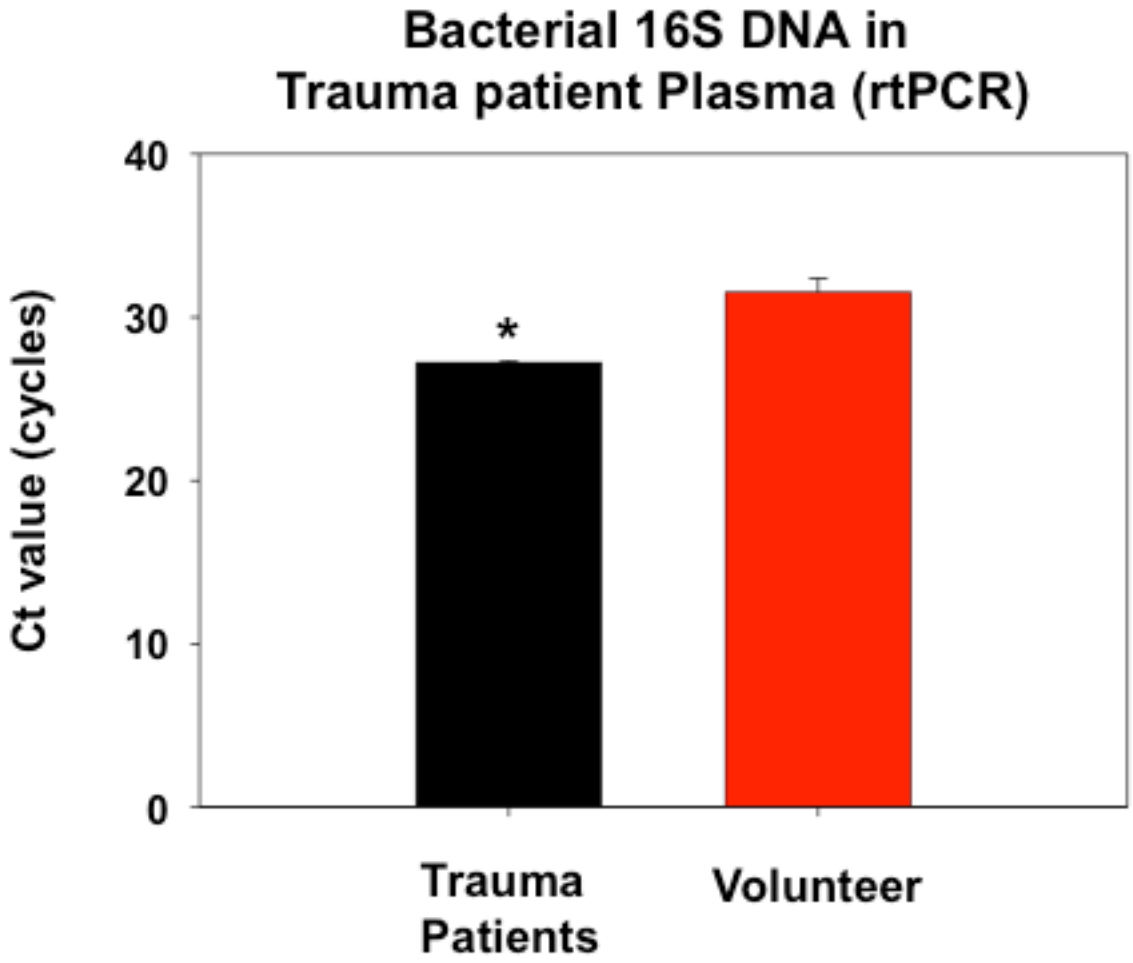
Fig. 4. Alveolar permeability was increased by PC. Permeability in the PC+5%MT group increased significantly, but less than it did in the absence of 5% MT IP (n=5-8, *P<0. 05 versus control, $, P< 0.05 versus 5%MT IP, ANOVA/ Dunn).

Figure 5



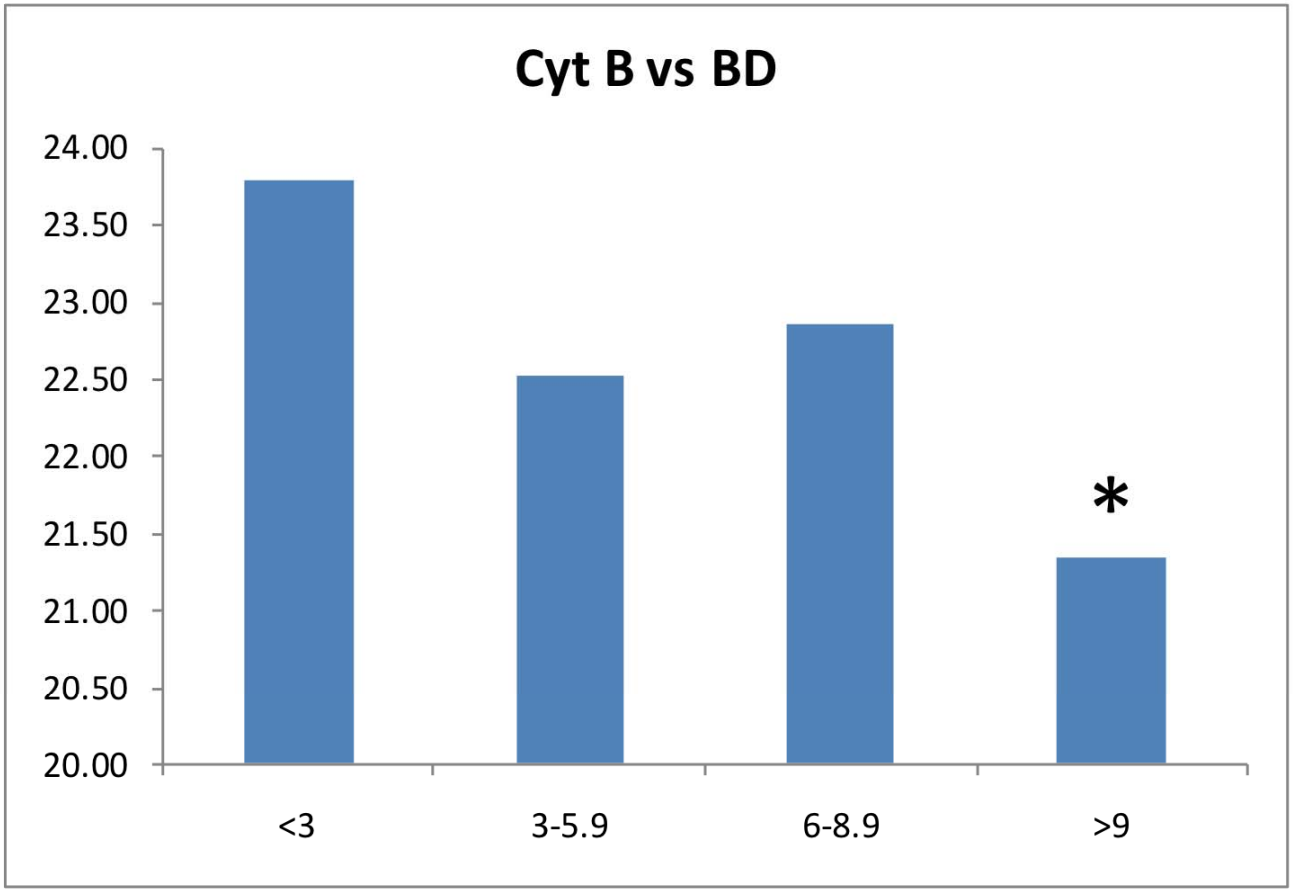
Trauma patients, N=100. Volunteers, N=12. All assays were run in duplicate. Mean +/- SE. P<0.001. Note DNA **increases** with lower Ct value.

Figure 6



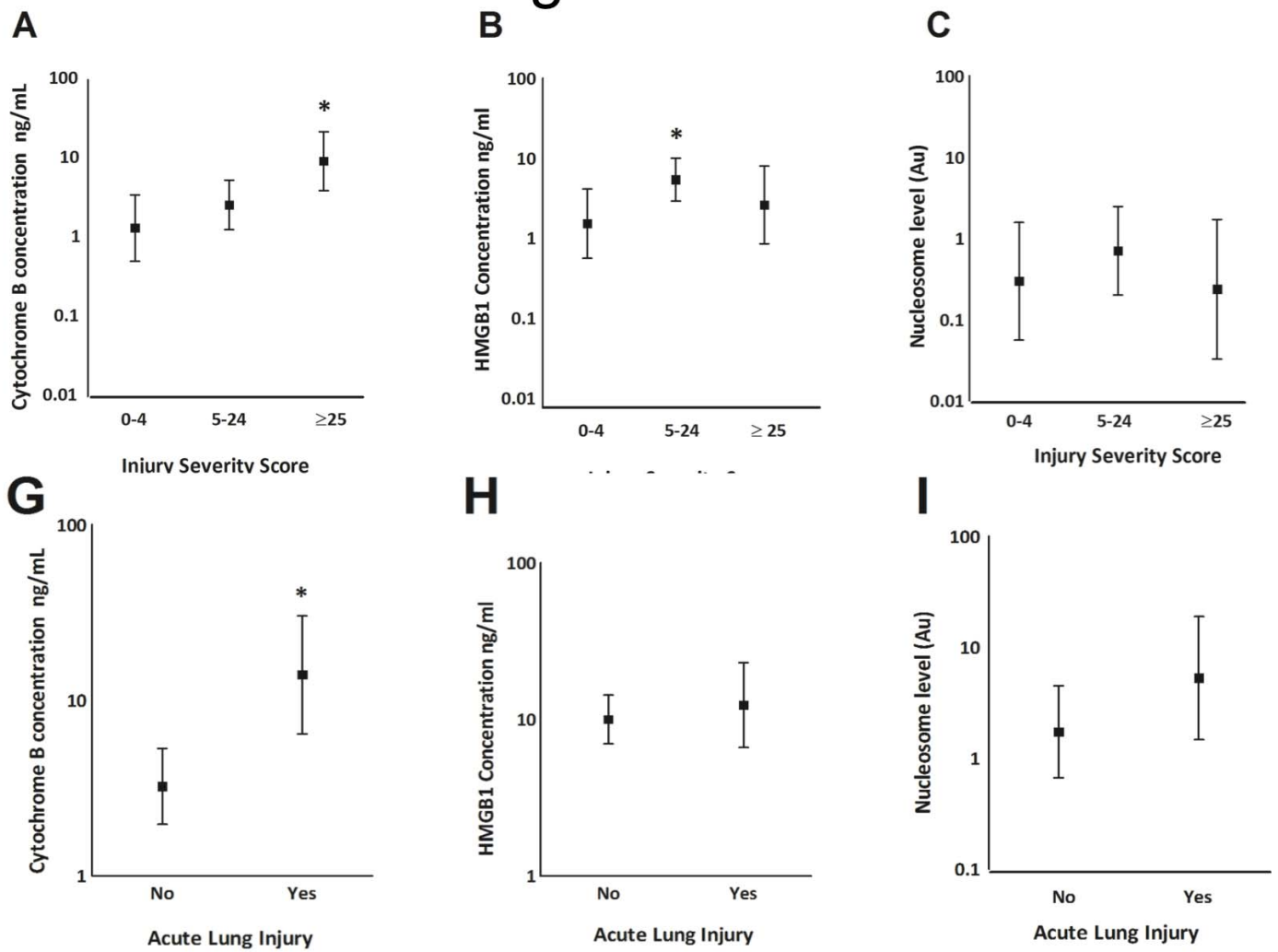
Trauma patients (N=100) had about 16-fold the plasma bDNA that volunteers (N=12) did. This was significant (P<0.04) but not nearly as much increase as that seen in mtDNA in the same patients

Figure 7



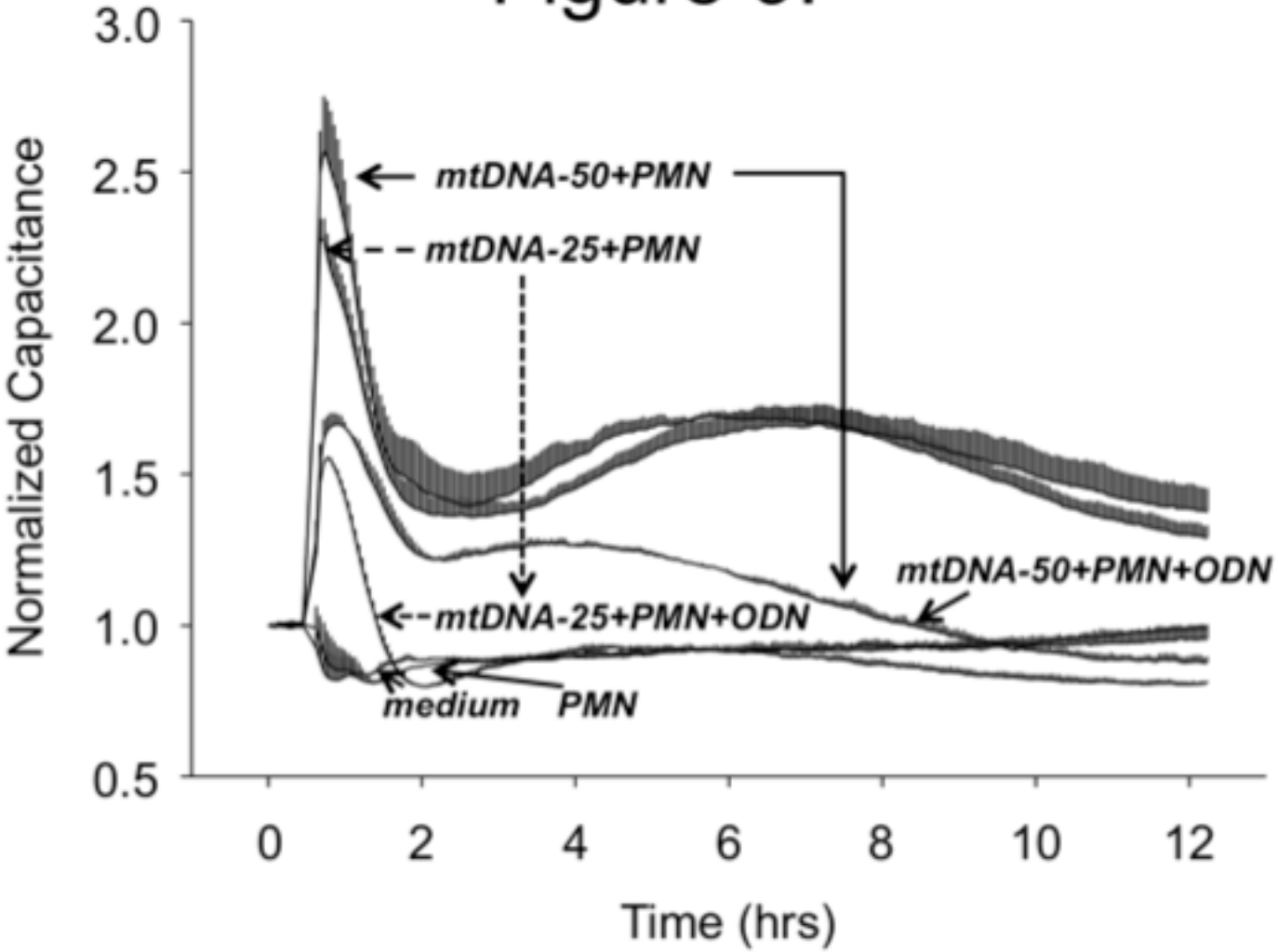
Regression of Cyt B versus Base Deficit
N=100, p=0.04

Figure 8



In regression analysis, increasing mtDNA is associated with increasing ISS (A) where other proposed DAMPs HMGB1 and histones (B, C) are not. mtDNA is also associated with acute lung injury (G) where HMGB1 and histones (B, C) are not (*P<0.01).

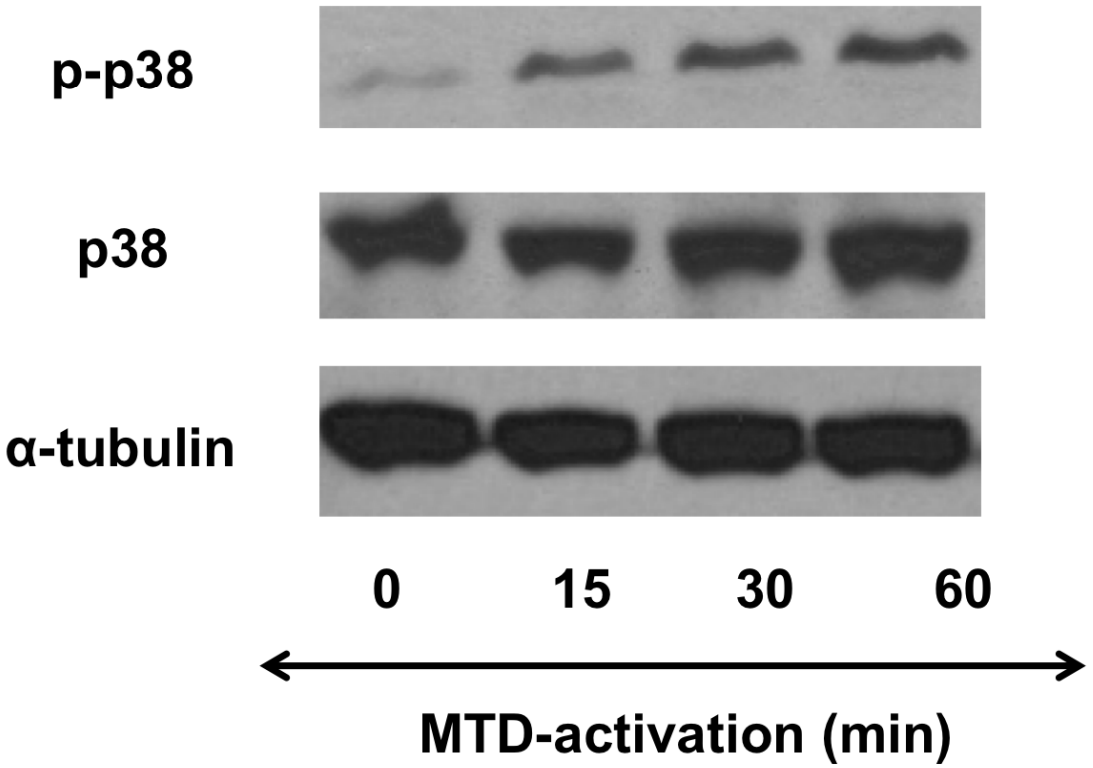
Figure 9.



Real-time permeability assay of endothelial cells seeded with PMN. Capacitance reflects monolayer permeability. PMN alone have no effect on EC, but in the presence of mtDNA cause marked permeability. This is reversed by inhibitory ODNs (see text)

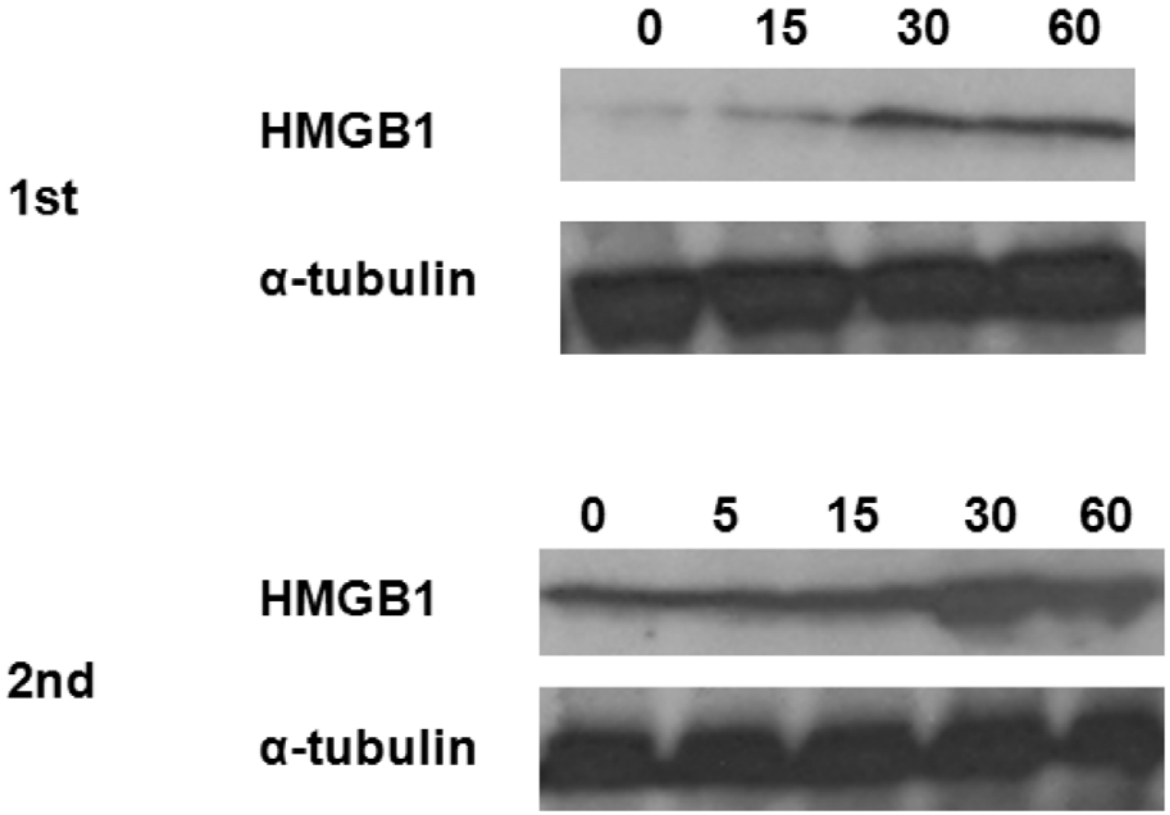
Figure 10

MTD-induced P-38 MAPK activation



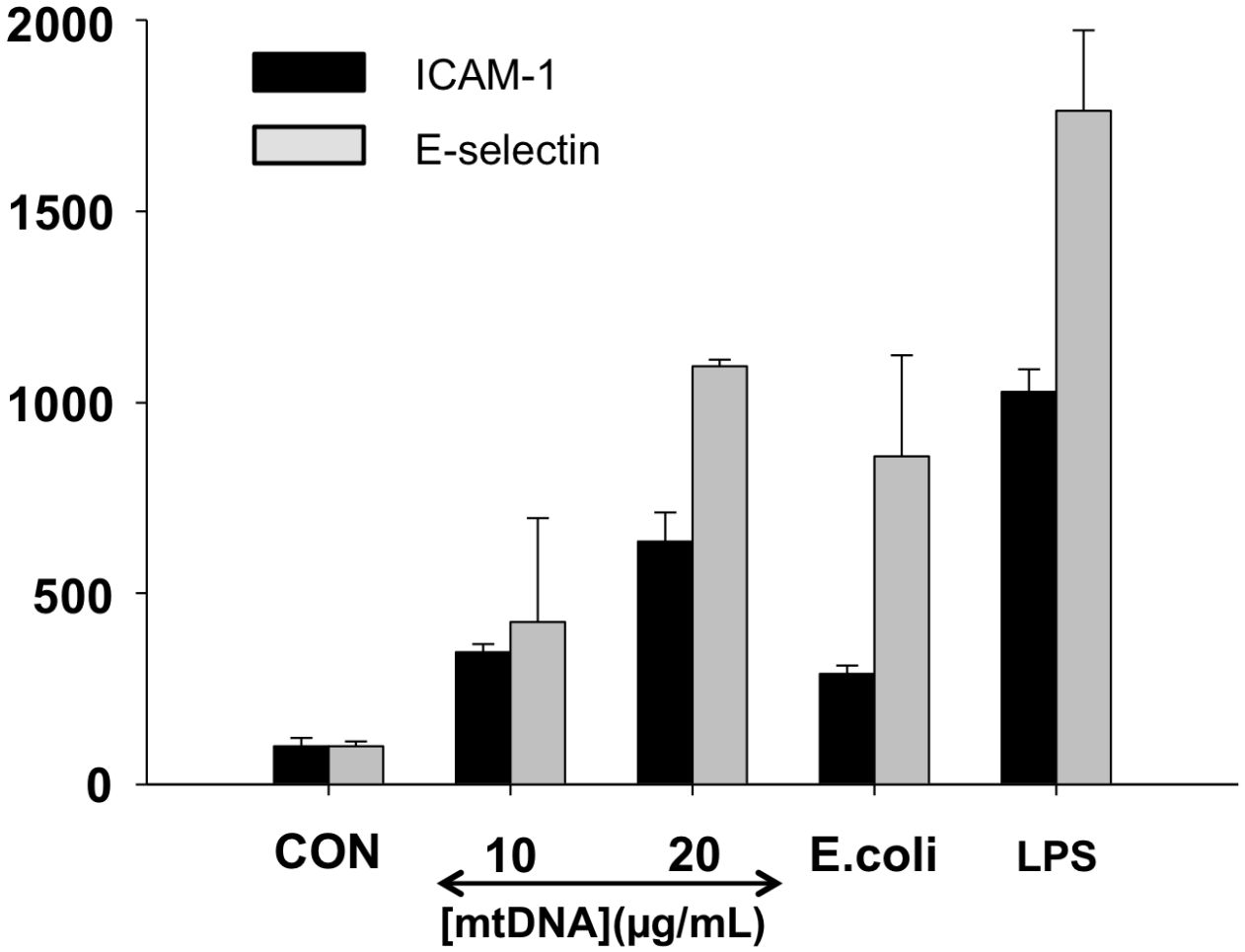
mtDNA induces rapid inflammatory activation of pulmonary artery endothelial cells (PAEC)

Figure 11

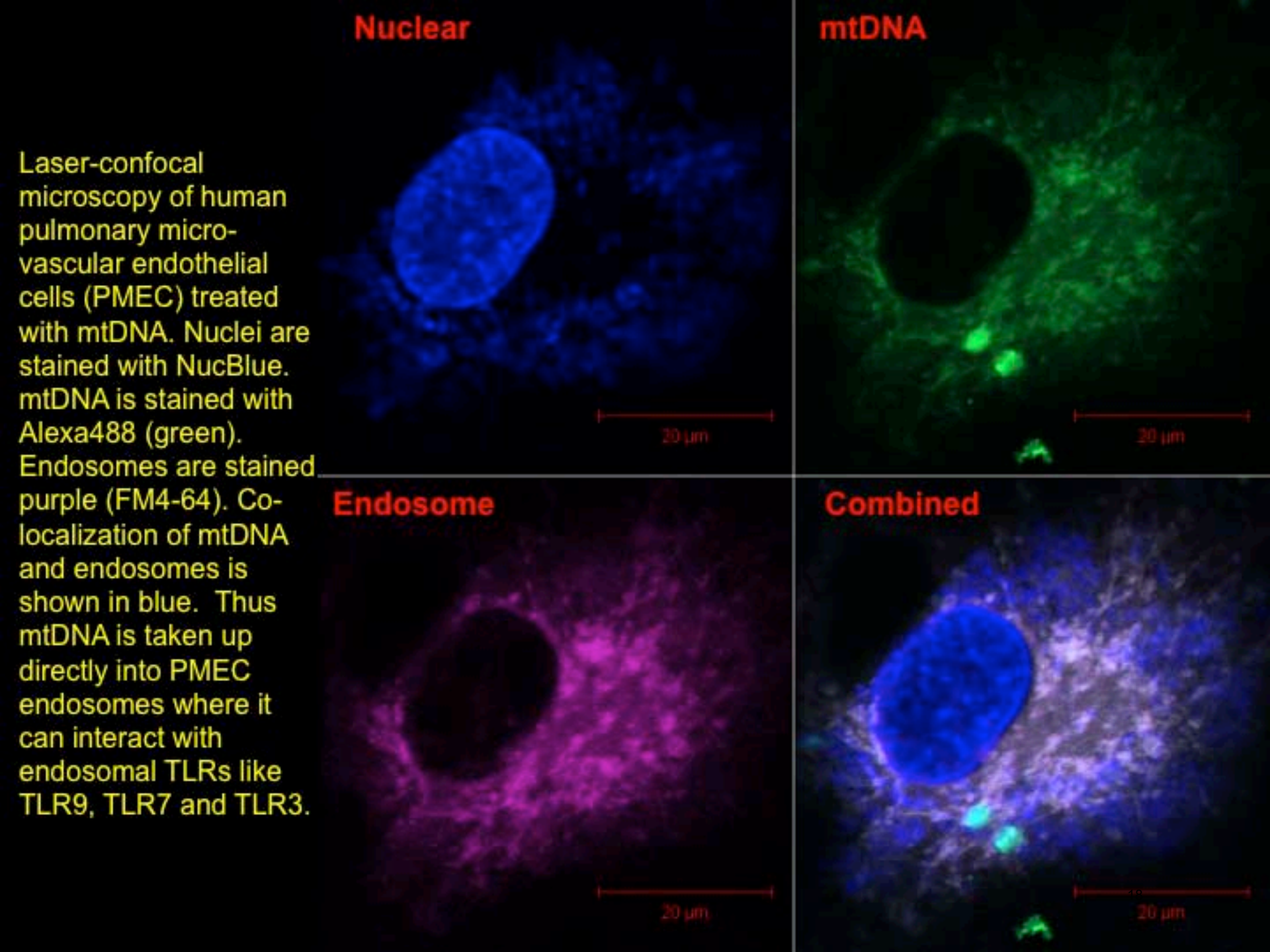


Human pulmonary microvascular endothelial cells (PMECs) stimulated by mitochondrial debris rapidly synthesize HMGB1. Western Blot, n=2.

Figure 12



mtDNA, E.Coli DNA and LPS treated EA cells, all show marked upregulation of adhesion molecules (qPCR)



Laser-confocal microscopy of human pulmonary micro-vascular endothelial cells (PMEC) treated with mtDNA. Nuclei are stained with NucBlue. mtDNA is stained with Alexa488 (green). Endosomes are stained purple (FM4-64). Co-localization of mtDNA and endosomes is shown in blue. Thus mtDNA is taken up directly into PMEC endosomes where it can interact with endosomal TLRs like TLR9, TLR7 and TLR3.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed sensitive and specific PCR primers for discrimination of sepsis and SIRS
- Validated the sensitivity and specificity primers in vitro
- Used this PCR-based approach to help understand the pathogenesis of SIRS in vitro.
- Used our PCR-based approach to help understand the pathogenesis of SIRS in patients with major fractures.
- Demonstrated proof of principle (using a baboon model) that our PCR-based analysis of mitochondrial and bacterial DNA will be useful in the assessment of sepsis and SIRS due to Anthrax and E. Coli.
- We have developed and perfected assays for mtDNA and bDNA. We have refined these tests to yield results in absolute amounts by creating reliable species-specific standards.
- We have created a relevant set of complex animal models to test injury-specific release of mitochondrial DAMPs as primary activators of innate immunity. These models include a chest concussion model to mimic blast injury that has been combined reproducibly with sepsis (CLP) and SIRS generated by mitochondrial injection. This will be an important future model in combat casualty research.
- We have demonstrated the critical role of mtDNA in the prolongation of SIRS after the resolution of sepsis and its link to lethality in an anthrax model.
- We have demonstrated that the presence of ***either*** distant infection ***or*** distant sterile injury (as mediated by mitochondrial DAMPs) does not act as a second-hit to worsen PMN mediated lung injury after trauma. Rather, distant “Danger” signals (as will be generated by blast or crush injuries or infection) act to divert PMN from the injured lung and make it susceptible to infection.
- We have done the first large scale study of mtDNA and bDNA circulation in human trauma patients.
- We have demonstrated the relationship between mtDNA circulation, SIRS and lung injury in a large trauma population.
- We have shown that mtDNA and MT-peptides are potent activators of PMN-endothelial interactions that lead to endothelial permeability. These are key elements in SIRS pathogenesis and therefore show conclusively that mitochondria are not simply ‘bystanders’ that are non-pathologic.
- We have uncovered important mechanisms by which mitochondria activate PMN-EC interactions and potentially contribute to end-stage sepsis by causing HMGB1 production and or release.

REPORTABLE OUTCOMES:

1) Publications:

1. Zhang, Q, Raoof, M, Sursal, T, Chen Y, Sumi Y, Junger W, Brohi K, Itagaki K and Hauser CJ. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* 464, 104–107 (2010)
2. Hauser CJ, Sursal T, Rodriguez EK, Appleton PT, Zhang Q and Itagaki, K. Mitochondrial DAMPs from femoral reamings activate neutrophils via formyl

peptide receptors and P44/42 MAP Kinase. J Orthop Trauma. 2010 Sep;24(9):534-8.

3. Raoof, M, Zhang, Q, Itagaki K and Hauser CJ. Mitochondrial Peptides Activate Neutrophils Via FPR-1. *J Trauma* 2010 Jun;68(6):1328-32.
4. Chen Y, Yao Y, Sumi Y, Li, A, To UK, Elkhal A, Inoue Y, Woehrle T, Zhang Q, Hauser CJ, Junger WG. Purinergic Signaling: A Fundamental Mechanism in Neutrophil Activation. *Science Signaling*, 2010 *in press.*
5. Itagaki K, Adibnia Y, Sun S, Zhao C, Sursal T, Chen Y, Junger W, Hauser CJ. Bacterial DNA induces pulmonary damage via TLR-9 through cross-talk with neutrophils. Shock, 2011, 36(6):548-52.
6. Sun S, Sursal T, Adibnia Y, Zhao C, Hauser CJ, and Itagaki K. Mitochondrial DAMPs Increase Endothelial Permeability through Neutrophil Dependent and Independent Pathways. PLoS ONE, 2012, submitted, in revision.
7. Sursal T, Stearns-Kurosawa, DJ Itagaki K, Oh, SY, Sun S, Kurosawa S, Hauser CJ. Plasma Bacterial and Mitochondrial DNA Distinguish Bacterial Sepsis from Sterile SIRS and Quantify Inflammatory Tissue Injury in Nonhuman Primates. Shock, ***in press***

2) Abstracts:

1. Itagaki K, Mu D, Zhang Q, Sursal T and Hauser CJ. PCR-based discrimination of sepsis from SIRS. Shock Volume 33, Suppl 1; P174 page 66, 2010.
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2010 Invited lecture: Modern concepts of sepsis. Region Sjaelland Danish Emergency Conference. Roskilde, Denmark, May 20-21, 2010.

2010 Invited lecture: Mitochondrial debris including mitochondrial DNA and formyl peptides appear in the blood after trauma inducing a syndrome

resembling sepsis. *Homo sapiens liberatus* workshop: Belozersky Institute, Moscow State University, Moscow, Russia. May 24-27, 2010.

2010 Plenary Address: The role of Mitochondrial DAMPs in recognition of tissue injury: Shock Society conference: The Role of Pattern Recognition Receptors in Non-Immune Cell Types in Shock and Sepsis. Portland OR, June 12-15, 2010.

2010 Invited lecture: Harvard Lung Conference: PAMPs, DAMPs and our evolving understanding of sepsis and SIRS. Harvard Medical School; Boston, MA. October 7, 2010.

2010 Plenary Address: The role of Mitochondrial DAMPs in recognition of tissue injury: Shock Society conference: The Role of Pattern Recognition Receptors in Non-Immune Cell Types in Shock and Sepsis. Portland OR, June 12-15, 2010.

2011 Western Trauma Association Founders lecture on Basic Science, March 3, 2011. Inflammation after Injury – sniffing the trail from femur fractures to formyl peptides.

2011 Distinguished Visiting Lecturer, University of Texas San Antonio, April 9-11, 2011. Surgery Grand Rounds Lecture: Inflammation after Injury – sniffing the trail from femur fractures to formyl peptides.

2011 Keynote Lecturer, American Thoracic Association, Denver Convention Center, May 16-16, 2011. DAMPs In Trauma-Induced SIRS.

2011 Invited Lecturer, Harvard Shock Symposium, Harvard Medical School, May 31, 2011. Mitochondrial DAMPs in Shock: Cause or Effect?

2011 Invited Keynote lecture: Mitochondrial DAMPs and the inflammatory response to tissue injury: ***Gordon Conference*** on Tissue Repair and Regeneration: Danger signaling: sensing the presence of damage. New London, NH, June 5-10, 2011.

2011 Distinguished Visiting Professor of Surgical Sciences: 10th Annual Department of Surgery Research Day. Emory University, Atlanta GA, June 16, 2011.

2011 Invited Lecture at American Society of Nephrology 44th Annual Meeting (November 10-13, 2011, Philadelphia, PA.) Lecture on Mitochondrial DAMPS and Inflammation after Injury for the Basic and Clinical Science Symposium “Innate immunity of kidney injury.”

2012 Memorial Regional Trauma Center 19th Annual Trauma Symposium. Hallandale beach, FL. Feb 9-11 2012. Sepsis and Inflammation: Molecular Biology 101 in Acute Trauma Care.

2012 Memorial Regional Trauma Center 19th Annual Trauma Symposium.

Hallandale beach, FL. Feb 9-11 2012. Catastrophic Abdominal Injuries: how to get out of trouble.

2012 Berkshire Medical Center 8th Annual Trauma & Critical Care Symposium, March 23, 2012. Invited lecture: PAMPs and DAMPs.

2012 Plenary Session moderator: Endogenous Triggers of the Injury Response. Shock Society: Miami Beach, FL, June 9-13, 2012.

2011 Surgical Horizons Seminar: "Danger" molecules as inflammatory agonists and biomarkers in sepsis and SIRS. December 12, 2011; Beth Israel Deaconess Medical Center, Boston, MA.

2010 Invited lecture: Mitochondrial debris including mitochondrial DNA and formyl peptides appear in the blood after trauma inducing a syndrome resembling sepsis. *Homo sapiens liberatus* workshop: Belozersky Institute, Moscow State University, Moscow, Russia. May 24-27, 2010.

2010 Invited Keynote address: New roles for Mitochondria in the Inflammatory Response to injury: Targeting Mitochondria 2010, Strategies, innovations & clinical applications**.** Berlin, Germany; November 18-19 2010.

2011 Sigma Tau Association lecture: University of Modena, Italy "Mitochondrial and Bacterial Molecular Motifs: Emerging Biomarkers for Sterile Inflammation and Sepsis." November 23, 2011.

2011 Sigma Tau Association lecture: Italian National Research Center, Naples, Italy. "Mitochondrial and Bacterial Molecular Motifs: Emerging Biomarkers for Sterile Inflammation and Sepsis." November 24, 2011.

4) Patents Applied For:

U.S. Provisional Application Serial No. 61/266,990: Discriminative Test for Infective Versus Sterile Inflammation

Degrees obtained that are supported by this award;
None

Development of cell lines, tissue or serum repositories:
None

infomatics such as databases and animal models, etc.;
None

Funding applied for based on work supported by this award;
Rib fractures, DAMPs and Pneumonia

R21 proposal by Dr. Kiyoshi Itagaki, submitted to National Institute on Aging, June 2012. This program will look at the role of mitochondrial DAMPS in pneumonia in the elderly after rib fractures.

- Employment or research opportunities applied for and/or received based on experience/training supported by this award

None

-Experience/training supported by this award

Masters program practical student experience in the lab (T. Sursal)

CONCLUSIONS:

In summary, we have been able to "leap-frog" ahead to achieve many of our specific aims using cellular, rodent, primate and human models. We have developed important, new and unexpected findings. The work done as part of this program has led to important collaborations and the development of new grant funding sources. It will certainly lead to crucial military and bio-defense applications in the quite near future.

This work has furthered the basic principle that mitochondria, because of their ancient status as archaebacteria, persist as critical initiators of the immune response to injury. In the case of the military combattant subjected to blast or crush injury, the implicationsa are critical: the destruction of tissue is a primary immune event that is at the center of our understanding of how wounding affects both inflammation and responses to infectious innoculation. Here we found out that mitochondria drive several key events in major injury. To study that we created multiple novel assays. Specifically, we found that release of mitochondria is at the heart of PMN-endothelial interactions that lead to organ dysfunction syndromes that are the major cause of ICU morbidity and death after injury. We have found that PCR studies for mtDNA and bacterial DNA can discriminate SIRS from injury and sepsis. We have also learned why SIRS persists after sepsis is controlled. We have demonstrated that these effects can be seen in large unselected populations. Probably most important we have surprisingly learned that the most pronounced early effect of mitochondria on innate immunity is to cause sequestration and dysfunction of PMN. This critical finding will drive future research in many related fields. Perhaps most promising is improving our understanding of clinical inflammation, such as the care of postoperative patients and elderly patients with fractures who have been assumed to be febrile and at risk for pneumonia because of "atelectasis", who likely simply suffer from hypoimmunity when their PMN are going to sites of injury, guided by chemotaxins form mitochondria, to participate in wound cleansing and healing. This knowledge will change the way in which surgical and Medical care is delivered for the forseeable future.

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LETTERS

Circulating mitochondrial DAMPs cause inflammatory responses to injury

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Injury causes a systemic inflammatory response syndrome (SIRS) that is clinically much like sepsis[1]. Microbial pathogen-associated molecular patterns (PAMPs) activate innate immunocytes through pattern recognition receptors[2]. Similarly, cellular injury can release endogenous 'damage'-associated molecular patterns (DAMPs) that activate innate immunity[3]. Mitochondria are evolutionary endosymbionts that were derived from bacteria[4] and so might bear bacterial molecular motifs. Here we show that injury releases mitochondrial DAMPs (MTDs) into the circulation with functionally important immune consequences. MTDs include formyl peptides and mitochondrial DNA. These activate human polymorphonuclear neutrophils (PMNs) through formyl peptide receptor-1 and Toll-like receptor (TLR) 9, respectively. MTDs promote PMN Ca^{2+} flux and phosphorylation of mitogen-activated protein (MAP) kinases, thus leading to PMN migration and degranulation *in vitro* and *in vivo*. Circulating MTDs can elicit neutrophil-mediated organ injury. Cellular disruption by trauma releases mitochondrial DAMPs with evolutionarily conserved similarities to bacterial PAMPs into the circulation. These signal through innate immune pathways identical to those activated in sepsis to create a sepsis-like state. The release of such mitochondrial 'enemies within' by cellular injury is a key link between trauma, inflammation and SIRS.

Trauma is a leading cause of premature death[5]. Injury causes activation of PMNs, organ failure, susceptibility to infection and SIRS[1,6]. Bacterial translocation from ischaemic gut to circulation was long thought to cause SIRS[7]. This was disproven[8] although shock may cause gut inflammation[9]. Crushes and burns, however, cause SIRS without shock. Thus the molecular signals linking injury to inflammation remain unclear.

During infection, innate immunity is activated by PAMPs expressed on invading microorganisms. Pattern recognition receptors recognize PAMPs[2]. Bacterial proteins are *N*-formylated[10], so formyl peptides activate chemoattractant formyl-peptide receptors (FPRs). TLRs respond to many PAMPs, like bacterial DNA that stimulates TLR9. Because mitochondria evolved from saprophytic bacteria to endosymbionts to organelles, the mitochondrial genome (mtDNA) contains CpG DNA repeats and codes for formylated peptides[4,11]. Mechanical trauma disrupts cells, so we hypothesized injury might release mitochondrial DAMPs[3] into the circulation, activating immunity and initiating SIRS.

To prove trauma releases of MTDs into the circulation, we measured plasma mtDNA in 15 major trauma patients (Injury Severity Score[12] >25). Sampling was before resuscitation. Patients had no open wounds or gastrointestinal injuries (details in Supplementary Table 1). The mtDNA of the trauma patients was markedly elevated (Supplementary Fig. 1a–c) compared with volunteers (Supplementary Table 2). The mtDNA in trauma plasma was $2.7 \pm 0.94\ \mu g\ ml^{-1}$ (mean ± s.e.m.), thousands of fold higher than volunteers' levels (Supplementary Fig. 1d). The mtDNA was further elevated 24 h after injury (Supplementary Fig. 1e). Ultracentrifugates of reamed specimens obtained from femurs during clinical fracture repair contained even higher titres of mtDNA. Thus MTDs are mobilized by either external or operative

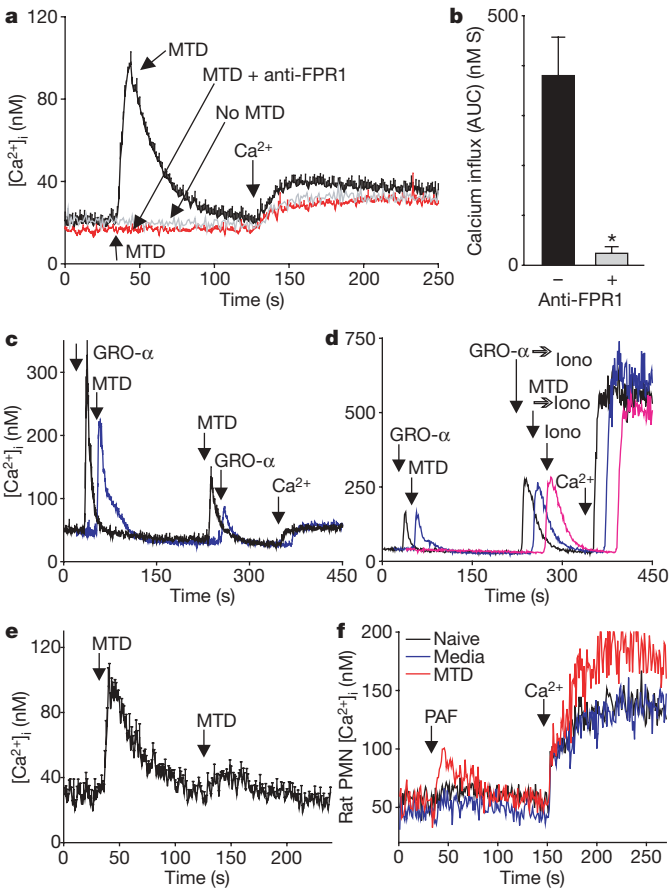

Figure 1 | PMN $[Ca^{2+}]_i$ responses to MTD. Rhabdomyosarcoma-derived MTD ($1.2\ \mu g\ ml^{-1}$ protein) induces Ca^{2+} store depletion (**a**) and Ca^{2+} influx (**a**, **b**) in human PMNs (*$P = 0.01$, *t*-test). **c**, PMN serially exposed to MTD and GRO-α (CXCL1) exhibit heterologous desensitization. **d**, PMN stimulated with GRO-α or MTD show equal store release by ionomycin (Iono). **e**, PMN show homologous desensitization of $[Ca^{2+}]_i$ responses to MTD. **f**, Systemic injection of MTD increases rat PMN responses to PAF. Traces with error bars are from at least three experiments; other traces are exemplary. Traces may be displaced temporally for clarity.

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injury to enter the circulation. Bacterial 16S RNA was absent from all specimens (Supplementary Fig. 1f).

Mitochondrial formyl peptides can attract PMNs[13] and activate related cell lines[14]. The synthetic peptide *N*-formyl-Met-Leu-Phe (fMLF) simulates bacterial challenge. However, the role of endogenous formyl peptides in trauma, PMN activation and SIRS is unstudied. Formyl peptides signal through the G-protein-coupled receptors FPR1 and FPRL-1, with high and low affinities respectively. PMN activation by G-protein-coupled receptors causes increased intracellular calcium ($[Ca^{2+}]_i$)[15], heterologous and homologous G-protein-coupled receptor desensitization[16] and activates MAP kinases (MAPKs)[17]. MTD from human myocytes induced human PMN $[Ca^{2+}]_i$ fluxes equal to 1 nM fMLF (Fig. 1a). MTD from human liver, muscle and fracture haematoma (Supplementary Fig. 2a) or from rat muscle or liver produced similar PMN Ca^{2+} depletion. Whole and fragmented mitochondria had similar potency (Supplementary Fig. 2b). Thus release of MTD from all cell types studied activates immunity.

Blocking antibodies to FPR1 abolished Ca^{2+} depletion (Fig. 1a) and Ca^{2+} entry (Fig. 1b) responses to MTD. Cyclosporin H (CsH) inhibits FPR1[18] and abolishes Ca^{2+} flux to MTD (Supplementary Fig. 3a). Isotype control (FPRL-1, matrix metalloproteinase (MMP)-2) antibodies have no effects (Supplementary Fig. 3b). Apyrase-treated and untreated MTDs act identically whereas apyrase abolishes $[Ca^{2+}]_i$ response to ATP (Supplementary Fig. 3c). ATP was undetectable on random assays of MTD ($n = 3$).

Activating FPR1 desensitizes chemokine receptors, predisposing to infection after trauma[16]. Human PMNs treated with MTD became insensitive to GRO-α (CXCL1, Fig. 1c). PMNs stimulated by GRO-α, MTD or buffer (Fig. 1d) show identical Ca^{2+} release by ionomycin. Because Ca^{2+} stores are equal, suppression by MTD reflects CXCR2 desensitization by FPR1. PMNs also show homologous desensitization when re-challenged with MTD (Fig. 1e) or fMLF (Supplementary Fig. 4). Others have shown that PMN MAPKs are phosphorylated and activated by injury[17]. Skeletal muscle MTD caused phosphorylation of PMN p38 and p44/42 MAPKs (Fig. 2a, b) with p38 being activated at lower concentrations. Thus muscular injury can liberate mitochondrial DAMPs that activate multiple inflammatory signal pathways.

Because mitochondrial DAMPs activated PMN signalling we studied whether they elicit an inflammatory PMN phenotype. MMP-8 is a neutrophil-specific collagenase[19] that aids in PMN tissue penetration and recruitment. Interleukin (IL)-8 causes PMN chemotaxis and activation, and such PMN activation also induces secondary IL-8 release. MTD caused MMP-8 release from human PMNs (Fig. 2c). Inhibition by CsH or anti-FPR1 again demonstrates FPR1 dependence (Fig. 2d). Human PMNs synthesized and released IL-8 in response to MTD (Fig. 2e, f) more rapidly than to LPS. This 'bell-shaped' response curve (Fig. 2e) may reflect FPR1 suppression by high concentrations of MTD (see Fig. 1e). In longer incubation studies, LPS was more potent (Fig. 2f).

PMNs use lytic enzymes like MMPs to migrate into bystander organs. We assessed the effects of MTD on PMN migration. Under video-microscopy, PMNs migrated towards MTD from clinical femur fractures (Fig. 2g–j and Supplementary Videos 1–4). Speed and direction of migration were inhibited by CsH[18] (Fig. 2i, Supplementary

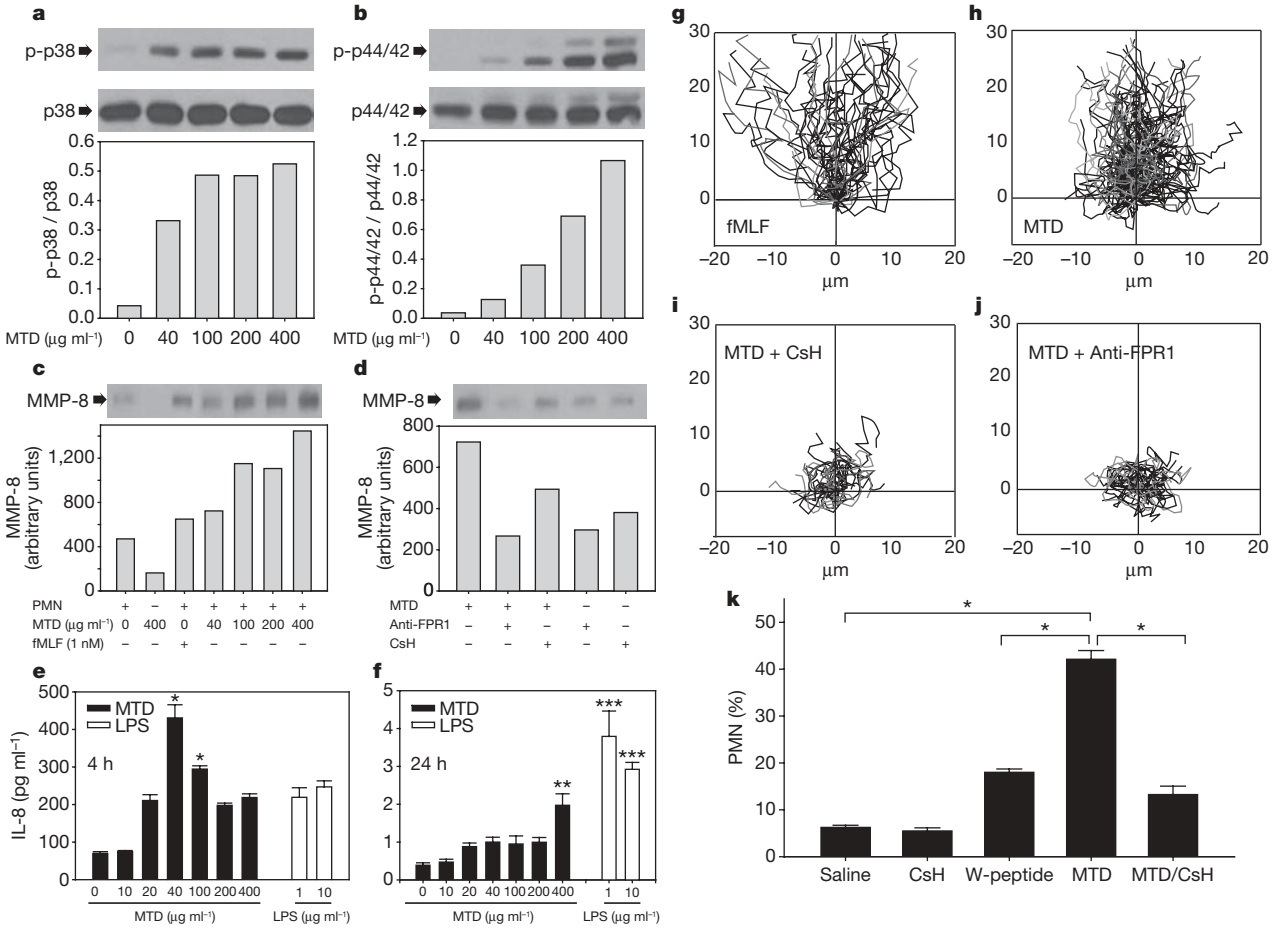

Figure 2 | MTDs activate PMNs. Human PMNs exposed to human MTDs (muscle) were immunoblotted for phosphorylated and total (control) p38 (**a**) or p44/42 MAPK (**b**). MMP-8 was immunoblotted in supernatants (**c**, **d** are from same gel). **e**, **f**, MTD elicits PMN IL-8 synthesis: */**$P < 0.05$ compared with control; ***$P < 0.05$ (analysis of variance/Tukey) compared with control or MTD ($n = 3$). PMN chemotaxis to fMLF and MTD was analysed by video-microscopy (**g**, **h** and Supplementary Videos 1 and 2). CsH or anti-FPR1 drastically inhibit chemotaxis (**i**, **j** and Supplementary Videos 3 and 4). MTD injection into the mouse peritoneum (**k**) causes rapid CsH-inhibitable neutrophil influx ($n = 6$, *$P < 0.05$, analysis of variance/Dunn) compared with saline or 10 nM W-peptide controls.

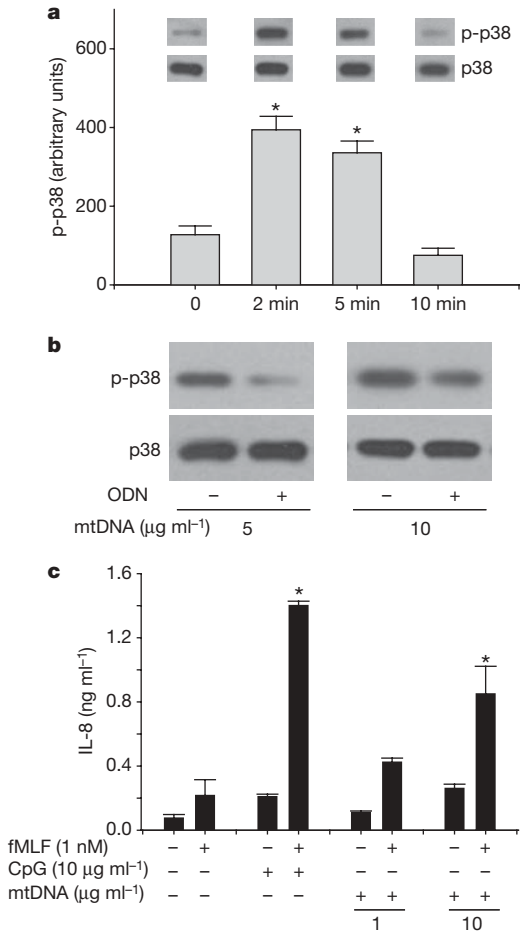

Figure 3 | Mitochondrial DNA activates PMN through CpG/TLR9 interactions. **a**, Incubation of PMN (10^6) with 1 μg ml^{-1} mtDNA activates p38 MAPK ($n = 3$, $^*P < 0.05$ compared with unstimulated cells). **b**, Activation of p38 MAPK induced by mtDNA was inhibited by pre-treatment with the inhibitory oligodeoxynucleotide TTAGGG. Inhibition was overcome at higher mtDNA concentrations. **c**, PMNs were co-incubated in 1 nM fMLF plus mtDNA at clinical concentrations (1–10 μg ml^{-1}, see Supplementary Fig. 1d). Neither CpG DNA nor mtDNA caused IL-8 release alone, but each caused significant release with low-dose fMLF ($n = 3$, $^*P < 0.05$ compared with unstimulated control). (All tests analysis of variance/Dunn.)

Video 3) or by antibodies to FPR1 (Fig. 2j and Supplementary Video 4). Last, we showed *in vivo* PMN infiltration in response to clinical concentrations of MTD by placing enough liver-derived MTD into mouse peritoneum to model traumatic necrosis of 10% of the mouse's liver. Neutrophilic peritonitis developed quickly (Fig. 2k). MTD was more active than the FPR agonist W-peptide, and CsH again reduced peritonitis (Fig. 2k).

Mitochondria contain their own genome, but mtDNA resembles bacterial DNA in being circular and having non-methylated CpG motifs[20]. Mitochondrial DNA has been found in fluids in joints in rheumatoid arthritis and induces inflammation *in vivo*[21]. CpG DNA activates TLR9 but activation of PMN by mtDNA is unstudied. TLR9 is expressed by PMN[22] and activates p38 MAPK[23]. So we questioned whether PMN p38 MAPK would be activated by mtDNA at clinical plasma concentrations (Supplementary Fig. 1d). We found 1 μg ml^{-1} mtDNA caused p38 MAPK phosphorylation (Fig. 3a) but did not activate p44/42 MAPK. Activation of p38 MAPK was blocked by inhibitory oligodeoxynucleotides (TTAGGG, Fig. 3b) that bind CpG motifs and block interactions with TLR9. Looking at downstream signalling, we incubated PMN with CpG DNA (10 μg ml^{-1}) or mtDNA within the clinical range (1–10 μg ml^{-1}). Neither released IL-8 effectively alone, but each promoted IL-8 release with low-dose fMLF (1 nM) (Fig. 3c). This is similar to granulocyte–macrophage

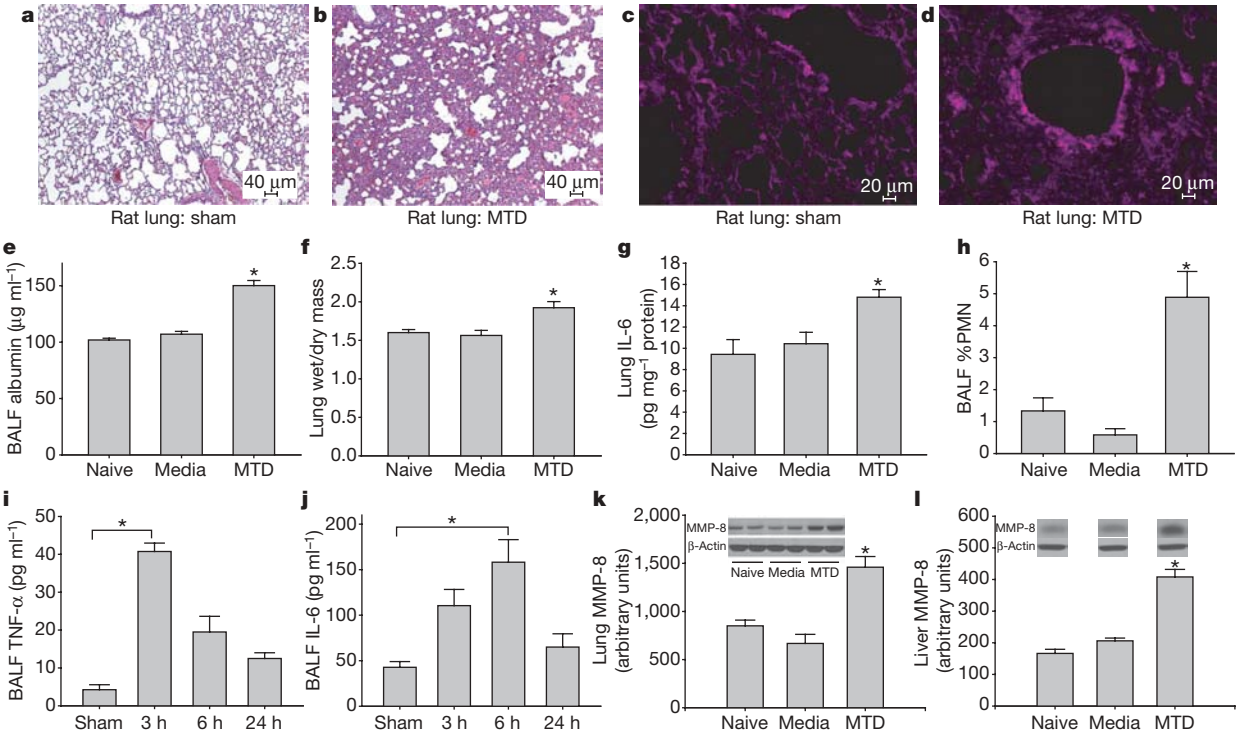

Figure 4 | MTDs cause systemic inflammation and organ injury *in vivo*. Rats given intravenous MTDs equivalent to mitochondria from a 5% liver injury exhibit marked evidence of lung injury, as shown by haematoxylin and eosin histology (**a**, **b**) and 4-hydroxy-2-nonenal stain for oxidant injury (**c**, **d**). MTDs increased pulmonary albumin permeability (**e**), lung wet/dry mass (**f**), accumulation of IL-6 in lung (**g**) and PMN infiltration into the airways (**h**). Early (3 h) appearance of tumour-necrosis factor-α (**i**) and late (6 h) appearance of IL-6 (**j**) were noted in lung lavage fluid. Whole lung (**k**) and liver (**l**) MMP-8 confirmed increased PMN infiltration. (All studies $n \geq 3$, $^*P < 0.05$, analysis of variance/post hoc.)

colony-stimulating factor priming of IL-8 release by CpG DNA[22]. These data suggest clinically significant activation of PMN secretion by mtDNA/TLR9. In distinction, TLR ligands have no direct effect on PMN chemotaxis (Supplementary Fig. 5).

To determine whether circulating mitochondrial DAMPs could cause neutrophil-mediated organ injury, we injected MTDs equivalent to 5% of the rat's liver intravenously and examined whether that recreated organ injury *in vivo*. Animals demonstrated marked inflammatory lung injury as early as 3 h after injection (compare Fig. 4a, b). Oxidant lung injury was documented by staining for 4-hydroxy-2-nonenal[24] (compare Fig. 4c, d). MTD injection increased lung albumin (Fig. 4e) and wet/dry mass (Fig. 4f), IL-6 (Fig. 4g) as well as elastase accumulation in lung (Supplementary Fig. 6). Bronchoalveolar lavage showed PMN influx into the airways (Fig. 4h), early appearance of tumour-necrosis factor-α (Fig. 4i) and later appearance of IL-6 (Fig. 4j). PMN infiltration was confirmed as increased lung MMP-8 (Fig. 4k). Systemic inflammation was demonstrated as priming of circulating PMNs (Fig. 1f) and their infiltration into liver (Fig. 4l). Control rats showed no evidence of pulmonary or hepatic inflammation.

In conclusion, inflammation occurs after both major trauma and infection[16]. Recognizing sterile SIRS is critical because empiric antimicrobial use will be ineffective whereas other therapies might be effective. After tissue trauma, MTD circulates and stimulates PMNs, causing systemic inflammation. The molecular similarity of mitochondria to their bacterial ancestors helps explain why traumatic and infective SIRS appear similar[3,25]. Mitochondrial DAMPs express at least two molecular signatures (formyl peptides, mtDNA) that act on pattern recognition receptors recognizing bacterial PAMPs. These activate PMN in the circulation (Figs 1f, 2 and 3) rather than at specific targets, inciting non-specific organ attack (Fig. 4) while suppressing chemotactic responses to infective stimuli (Fig. 1c, e and Supplementary Fig. 4).

Formyl peptides and mtDNA are likely only a subset of the DAMPs released by trauma, but they appear important at clinical concentrations. Other intracellular 'alarmins' may similarly be important after injury, and other immune cells probably respond to mitochondrial DAMPs. Injury-derived mitochondrial DAMPs, however, are clearly recognized by innate immunity using pattern recognition receptors that alternatively sense bacteria. This novel model may explain why responses to these ancient 'enemies within' released by injury can mimic sepsis.

METHODS SUMMARY

All studies were approved by the institutional review boards of Beth Israel Deaconess Medical Center, Boston, USA, and Queen Mary's University Hospital, London, UK. Animal care was approved by the Institutional Animal Care and Use Committee according to National Institutes of Health guidelines.

Preparation of mitochondria, MTDs and mtDNA. Mitochondria were isolated from resources as per standard protocols.

PMN studies. PMN isolation[26,27], calcium studies[15,16,26], western blots[28], transwell chemotaxis[16] and video-microscopy chemotaxis assays[29] were performed as previously described.

MTD administration. Male Sprague–Dawley rats were given intravenous MTDs based on mass[30]. Quantitative PCR of plasma showed mtDNA levels of $122 \pm 22\ \mathrm{ng\,ml^{-1}}$ 1 h after injection (normal levels are very much less than $1\ \mathrm{ng\,ml^{-1}}$). Leukocytes in bronchoalveolar lavages were counted visually. Lungs were inflated gently and fixed in formalin before staining with haematoxylin and eosin or for 4-hydroxy-2-nonenal.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Reagents and chemicals. fMLF, EGTA, protease inhibitor cocktail and DMSO were purchased from Sigma. Fura-2 AM, calcein AM and digitonin were purchased from Molecular Probes. Anti-human FPR1, anti-human FPRL-1, anti-human MMP-2, anti-human MMP-8 and anti-rat MMP-8 antibodies were purchased from R&D. Antibodies to phospho-p38 MAPK (Thr 180/Tyr 182), p38 MAPK, phospho-p44/42 MAPK (Thr 202/Tyr 204) and p44/42 MAPK were from Cell Signaling. Donkey anti-goat IgG-HRP was obtained from Santa Cruz. ImmunoPure Goat Anti-Rabbit IgG (peroxidase conjugated) was purchased from Pierce Biotechnology. CsH was obtained from LKT Laboratories. ATP bioluminescence assay kits were purchased from Roche. W-peptide was from Phoenix Pharmaceuticals. CpG DNA was obtained from Cell Sciences. Oligodeoxynucleotide TTAGGG was purchased from InvivoGen.

Mitochondrial isolation from clinical material. Clinical liver injury, muscle crush injury and femur fracture fixation by reamed nailing are all common, important events closely linked to inflammation and acute lung injury after injury. The Mitochondria Isolation Kit for Tissue (Pierce) was used to isolate mitochondria from rat liver or rat muscle, human skeletal muscle (pathological specimens amputated owing to vascular disease); human femur medullary reamings from patients undergoing repair of femur fractures; and human liver from the margins of hepatic tumour resections. The Mitochondria Isolation Kit for Cultured Cells (Pierce) was used to isolate mitochondria from human rhabdomyosarcoma cells (ATCC). Clinical samples used to prepare mitochondria were harvested from patients receiving antibiotics. Mitochondria were isolated under sterile conditions at 4 °C.

Preparation of MTDs and mtDNA. Isolated mitochondrial pellets from tissue specimens (200 mg) or rhabdomyosarcoma cells (6×10^7 cells) were suspended in 1 ml of HBSS. Protease inhibitor cocktail (1:100) was added to the suspension. Because we found significant amounts of circulating mtDNA in trauma patients, we surmised that mechanical tissue injury and/or tissue necrosis was disrupting mitochondria to some extent *in vivo*. So we standardized our experimental preparations with routine sonication on ice (VCX130-Vibra Cell, Sonics and Materials) at 100% amplitude (ten times, 30 s each time with 30-s intervals). The disrupted mitochondrial suspensions were then centrifuged at 12,000*g* for 10 min at 4 °C followed by 100,000*g* at 4 °C for 30 min. Residual supernatants were used for experiments. Protein concentrations of the MTD solutions were determined by BCA Protein Assay (Pierce). Mitochondrial DNA was extracted from the isolated mitochondria of various tissues using DNeasy Blood & Tissue kit (Qiagen). MTDs and mtDNA were prepared under sterile conditions. Endotoxin levels were measured by Limulus amoebocyte lysate assay and did not achieve significant levels. Mitochondrial DNA concentration was determined by spectrophotometer. No protein contamination was found and nuclear DNA was less than 0.01% by quantitative PCR.

Real-time PCR protocols. Plasma DNA was prepared by QIAamp DNA Mini and Blood Mini kit (Qiagen). Primers for human cytochrome B (forward 5′-ATGACCCCAATACGCAAAAT-3′ and reverse 5′-CGAAGTTTCATCATGC GGAG-3′), human cytochrome C oxidase subunit III (forward 5′-ATGACCC ACCAATCACATGC-3′ and reverse 5′-ATCACATGGCTAGGCCGGAG-3′), human NADH dehydrogenase (forward 5′-ATACCCATGGCCAACCTCCT-3′ and reverse 5′-GGGCCTTTGCGTAGTTGTAT-3′), rat cytochrome B (forward 5′-TCCACTTCATCCTCCCATTC-3′ and reverse 5′-CTGCGTCGGAGTTTAA TCCT-3′), rat cytochrome C oxidase subunit III (forward 5′-ACATACCA AGGCCACCAAC-3′ and reverse 5′-CAGAAAAATCCGGCAAAGAA-3′), rat NADH dehydrogenase (forward 5′-CAATACCCCACCCCCTTATC-3′ and reverse 5′-GAGGCTCATCCCGATCATAG-3′) and bacterial 16S ribosomal RNA (forward 5′-CGTCAGCTCGTGTTGTGAAA-3′ and reverse 5′-GGC AGTCTCCTTGAGTTCC-3′) were synthesized by Invitrogen. Primer sequences have no significant homology with DNA found in any bacterial species published on BLAST. Real-time PCR standard curves were created to quantify mtDNA concentration by using purified mtDNA and cytochrome B as targets. Samples that produced no PCR products after 40 cycles were considered 'undetectable' and the Ct number set to 40 for statistical purposes.

PMN isolation. Detailed protocols are published elsewhere[26,27]. Hypotonic lysis was performed on ice to remove contaminating RBC. This method results in preparations containing at least 98% neutrophils and about 0.02% monocytes as assessed by flow cytometry and confirmed visually on HEMA-3 stain (Supplementary Fig. 7). Viability was at least 98% as assessed by Trypan blue.

Chemotaxis assays by fluorescence video-microscopy. Time-lapse video-microscopic chemotaxis was assayed as described previously[29]. Cells were exposed to a chemoattractant gradient field by slowly releasing MTD ($\sim$100 μg ml^{-1}) or fMLF (10 nM) from a micropipette tip placed in proximity to the cells. The migration paths of individual cells were plotted using Adobe Illustrator. Cells were pretreated with or without 1 μM CsH for 5 min or 12.5 μg ml^{-1} anti-human-FPR1 for 10 min. Experiments were repeated with multiple PMN and MTD isolates. Specificity of CsH for FPR1 was examined in transwell chemotaxis assays. CsH significantly inhibited fMLF and MTD chemotaxis with no effects on IL-8.

***In vivo* chemotaxis.** Male mice (8–10 weeks, Charles River) were used in this study. Mice were lightly anaesthetized by isoflurane inhalation. CsH (10 μM) or DMSO was injected intraperitoneally. After 30 min, 1 ml of saline or W-peptide (10 nM) or MTD (100 μg ml^{-1}, equal to the mitochondria released by a 10% liver injury) was injected intraperitoneally. Two hours later a peritoneal lavage was performed and collected for total and differential cell counts. Cell counts were performed on cytospin preparations stained with HEMA 3 (Fisher Scientific).

PMN degranulation assays. PMN degranulation was assessed by measuring MMP-8 release. Human PMN were suspended in HBSS with 1.8 mM Ca^{2+} at 37 °C for 10 min while exposed to MTD, mtDNA or fMLF at indicated concentrations. For inhibitor studies, PMNs were pre-treated with CsH (10 μM, 5 min at 37 °C), anti-FPR1 antibody (12.5 μg ml^{-1}, 10 min at 37 °C or control antibodies as noted) or inhibitory oligodeoxynucleotide TTAGGG (10:1 inhibitor to stimulus). After stimulation, PMNs were placed on ice and centrifuged. Supernatants were then assayed for MMP-8 by western blot. Residual PMNs were lysed to assay for MAPKs.

Enzyme-linked immunosorbent assay. PMNs were treated with various agonists for 4 h or 24 h. IL-8 was measured by human CXCL8/IL-8 ELISA (R&D). Experiments were performed in triplicate. Tumour-necrosis factor-α and IL-6 in rat bronchoalveolar lavage fluid or lung were measured using BD OptEIA^{TM} rat tumour-necrosis factor and IL-6 ELISA sets (BD). Airway albumin was measured by rat albumin ELISA Quantitation Set (Bethyl).

Statistical analysis. Study data were assessed for statistical significance using Student's (unpaired) *t*-test or analysis of variance where appropriate using a SigmaStat program with post hoc tests chosen by the computer. $[Ca^{2+}]_i$ transients are reported as the mean change from basal $[Ca^{2+}]_i$ in nanomoles per litre. Prolonged $[Ca^{2+}]_i$ fluxes are reported as the area under the curve of measured change from basal $[Ca^{2+}]_i$ over the observation period (nanomole seconds). All data are reported as mean ± s.e.m. with significance accepted at $P < 0.05$.

Mitochondrial Damage Associated Molecular Patterns From Femoral Reamings Activate Neutrophils Through Formyl Peptide Receptors and P44/42 MAP Kinase

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Hypothesis: Fractures and femoral reaming are associated with lung injury. The mechanisms linking fractures and inflammation are unclear, but tissue disruption might release mitochondria. Mitochondria are evolutionarily derived from bacteria and contain "damage associated molecular patterns" like formylated peptides that can activate immunocytes. We therefore studied whether fracture reaming releases mitochondrial damage associated molecular patterns (MTD) and how MTD act on immune cells.

Methods: Femur fracture reamings (FFx) from 10 patients were spun to remove bone particulates. Supernatants were assayed for mitochondrial DNA. Mitochondria were isolated from the residual reaming slurry, sonicated, and spun at 12,000 g. The resultant MTD were assayed for their ability to cause neutrophil (PMN) Ca^{2+} transient production, p44/42 MAPK phosphorylation, interleukin-8 release, and matrix metalloproteinase-9 release with and without formyl peptide receptor-1 blockade. Rats were injected with MTD and whole lung assayed for p44/42 activation.

Results: Mitochondrial DNA appears at many thousand-fold normal plasma levels in FFx and at intermediate levels in patients' plasma, suggesting release from fracture to plasma. FFx MTD caused brisk PMN Ca^{2+} flux, activated PMN p44/42 MAPK, and caused PMN release of interleukin-8 and matrix metalloproteinase-9. Responses to MTD were inhibited by formyl peptide receptor-1 blockade using cyclosporine H and anti formyl peptide receptor-1. MTD injection caused P44/42 phosphorylation in rat lung.

Conclusions: FFx reaming releases mitochondria into the wound and circulation. MTD then activates PMN. Release of damage signals like MTD from FFx may underlie activation of the cytokine cascades known to be associated with fracture fixation and lung injury.

Key Words: innate immunity, formyl peptides, fractures, neutrophils

(*J Orthop Trauma* 2010;0:000–000)

INTRODUCTION

Acute lung injury and adult respiratory distress syndrome (ALI/ARDS) occur after fractures in a sporadic entity often termed "fat embolism syndrome." Fat embolism syndrome is hard to distinguish from ALI/ARDS occurring after sepsis and may be associated with reamed nailing more than other methods of fixation. Current concepts emphasize that fracture hematomas are rich in inflammatory mediators[1–4] that can activate immune cells like neutrophils (PMN) that can injure the lung, but it is unknown what the primary events are causing fractures to be rich in mediators. Understanding the events linking mechanical injury to immune organ dysfunction is essential if effective therapies are to be developed.

Bacteria can cause inflammation by releasing lipids (like lipopolysaccharide) or *n*-formyl peptides.[5] Such "pathogen-associated molecular patterns"[6] activate cells through "pattern-recognition receptors" like toll-like receptor-4 (responds to lipopolysaccharide) or the receptors formyl peptide receptor (FPR)-1 and FPRL-1 that respond to formyl peptides (FP).[7] Human genomic proteins are not formylated, but mitochondria resemble bacteria in many ways, having FP and circular DNA (mitochondrial DNA) with nonmethylated repeats like bacterial DNA.[5,8,9] These observations led to the conclusion that mitochondria were once free-living bacteria that became intracellular symbionts.[10,11] They also suggest that when mitochondrial molecular patterns are released from cells by injury, they might activate immunity by mimicking bacterial motifs. Intrinsic molecular motifs like this are referred to as "damage associated molecular patterns" (DAMPs)[12] or "alarmins." We have shown that mitochondrial FPs activate PMN[5] and that mitochondrial DNA is released in shock.[8] We now hypothesized that mitochondrial FP and other mitochondrial DAMPs (MTD) are released when cells are disrupted during fracture injury or reaming. If so, they could activate immunocytes and contribute to fracture-related lung injury.

MATERIALS AND METHODS

Research Compliance

Studies were performed under the supervision of the Institutional Review Board of Beth Israel Deaconess Medical Center and Harvard Medical School. Fracture reaming specimens were collected under waiver of consent for discarded materials. Consent was obtained for sampling and archiving of trauma plasma samples from the patients or their legally

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authorized representative whenever such consent was available. Animal experimentation was approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Patients and Biologic Samples

Femoral reamings were collected intraoperatively from 10 patients with diaphyseal femur fractures. Patients were 18 to 54 years old and had isolated closed injuries sustained in motor vehicle traumas. Specimens were kept at 4°C while processed. Specimens were spun to remove gross particulates. The residual cellular material was subjected to a standard mitochondrial isolation protocol using a kit (Pierce, Rockford, IL). Plasma specimens were obtained when consent for blood draw was available (n = 5). Mitochondria were sonicated and spun at 12,000 g. The supernatant was assessed for biologic activity of MTD. Samples of fracture fluid were also spun at 12,000 g and the supernatants assayed by quantitative polymerase chain reaction in triplicate to assay mitochondrial DNA.

Animals

Male Sprague-Dawley rats (250–350 g; Charles River, Wilmington, MA) were acclimatized under barrier sustained conditions (25ºC, 12-hour light/dark cycles, water and chow ad libitum). Animals were cannulated as per our published methods[13] and injected (n = 3/group) with MTD from human fracture hematomas or media. Animals were euthanized 1 hour later. Lungs were harvested and frozen at –80°C for later analysis.

Reagents and Chemicals

We purchased fMLP, EGTA, and DMSO from Sigma (St. Louis, MO); Fura2-AM from Molecular Probes (Eugene, OR); antihuman FPR1 from R&D (Minneapolis, MN); Hanks' balanced salt solution and phosphate-buffered saline from GIBCO (Grand Island, NY); antihuman matrix metalloproteinase-9 from Chemicon (Billerica, MA); and phospho-p44/42 MAP kinase (Thr202/Tyr204) and p44/42 MAP kinase antibodies from Cell Signaling (Danvers, MA).

Details of Mitochondrial Isolation

Mitochondria were isolated using a Mitochondria Isolation Kit for tissue (Pierce, Rockford, IL) according to the manufacturer's dounce-soft tissue protocol under sterile conditions at 4°C and then stored on ice for further processing.

Preparation of Mitochondrial Damage Associated Molecular Patterns

Mitochondria from 200 mg of tissue were suspended in 1 mL of buffer (Hanks' balanced salt solution for chemotaxis assays and HEPES for calcium assays). Protease inhibitor cocktail (1:100) was added. Suspensions were subjected to sonication on ice (VCX130-Vibra Cell; Sonics and Materials, Newtown, CT) at 100% amplitude three times for 30 seconds each. The disrupted mitochondria were centrifuged at 12,000 rpm for 10 minutes at 4°C. Supernatants were removed and stored at –20°C for experiments. Protein concentration was determined by BCA assay. We have previously noted that there are no interspecies differences in the responses to mitochondria between human and rat. Immune cells from rat and man respond equally to MTD from their own and the other species.[5,8,9]

Neutrophil Isolation

Detailed protocols are described elsewhere.[13,14] Briefly, heparinized volunteer blood is centrifuged and platelet-rich plasma removed. The buffy coat and 2 cm of red blood cells are layered onto Polymorphoprep gradient (Robbins Scientific, Sunnyvale, CA) and spun (1500 rpm, 30 minutes). PMNs are collected and osmolarity restored for 5 minutes with an equal volume of 0.45% NaCl. PMNs are washed, centrifuged, and a hypotonic lysis is done on ice to remove residual red blood cells. The preparation contains 98% or more neutrophils (cytospin) that are 99% or greater viable (Trypan blue). PMN pellets are resuspended in Hanks' balanced salt solution with 5% fetal bovine serum for chemotaxis assays or in HEPES buffer with 0.1% bovine serum albumin for calcium and oxidative burst experiments.

Calcium Dye Loading

PMNs were incubated in 2 μM fura-2AM (30 minutes, 37°C). Cells were divided into 200-μL aliquots and kept on ice. Aliquots were rewarmed to 37°C before experiments, centrifuged, resuspended in 200 μL of HEPES, and loaded into a cuvette containing 2.8 mL of the same buffer. Experiments were begun in "calcium-free" media (0.3 mM EGTA added) and 1.8 mM $CaCl_2$ was added to the media as indicated.

Spectrofluorometry

PMN $[Ca^{2+}]_i$ was measured using fura-2AM in a spectrofluorometer (Fluoromax-2; Jobin-Spex, Edison, NJ) using our modifications of the methods of Grynkiewicz et al.[15] Fracture fluids were added to the media at up to 20%. Higher concentrations of admixed protein solutions cause unacceptable autofluorescence.[14]

Western Blots

Rat lung homogenates, human PMN supernatants, and lysates were boiled for 5 minutes in sodium dodeclysulfate sample buffer. Proteins were separated by sodium dodeclysulfate polyacrylamide gel electrophoresis using 4% to 20% Tris-glycine polyacrylamide gradient gels (Novex, San Diego, CA). Separated proteins were transferred to nitrocellulose (0.45-μm pore size; Immobilon-P; Millipore, Bedford, MA). matrix metalloproteinase-9, Phospho-p44/42, and p44/42 MAPK were immunoblotted with specific antibodies and detected using enhanced chemiluminescence (Amersham, GE, Buckinghamshire, UK). Membranes were stripped and reprobed with β-actin (Santa Cruz, CA) to assess loading. Densitometry was performed with the Scion Image program. AU1

Quantitative Polymerase Chain Reaction

DNA was isolated from 200 μL of volunteer plasma as controls. DNA was isolated from the same volume of reaming fluid of patients with femur fractures or their plasma when available. DNA isolation was performed using QIAamp DNA Mini Kit (Qiagen). DNA concentration and quality was AU2 measured with a Nanodrop spectrophotometer. A total of 7.5 μL of DNA was used for each polymerase chain reaction.

SYBR green polymerase chain reaction was performed on volunteer plasma, patient plasma, and fracture fluid using primers targeting cytochrome B (CytB) that were specific (on "BLAST" analysis) for mitochondrial DNA. Primers (1R: CGAAGTTTCATCATGCGGAG and 1F: ATGACCCCAA-TACGCAAAAT) were synthesized by Invitrogen. AU3

Statistical Analyses

All $[Ca^{2+}]_i$ transient results reported are measured as the mean change from basal $[Ca^{2+}]_i$ in nanomoles per liter (nM). Quantitative polymerase chain reaction was assessed as cycle count number (Ct): increasing cycle number to detection reflects decreasing abundance of the target. Data were assessed for significance using Student's (unpaired) *t* test or analysis of variance where appropriate. Post hoc analyses for analysis of variance were selected by the SigmaStat program (Systat Software, Richmond, CA). Data are reported as the mean ± standard error of mean and statistical significance is accepted at a P value < 0.05. All examples of molecular studies shown are representative of three or more replications.

RESULTS

Mitochondrial Damage Associated Molecular Patterns in Fracture Patients

Mitochondrial DNA is a biomarker for the presence of MTD. We found that mitochondrial DNA was close to undetectable in volunteer plasma (Fig. 1). Fracture fluids contained approximately twofold[16] (greater than 100,000-fold) more mitochondrial DNA than volunteer plasma. Circulating plasma from patients with fractures had 500- to 1000-fold more mitochondrial DNA than volunteer plasma. F1

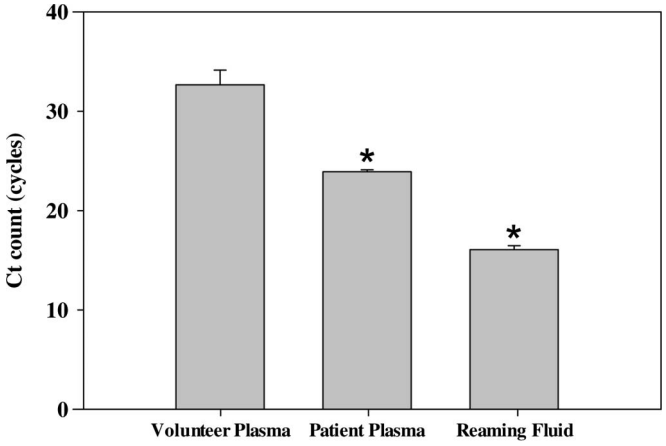

FIGURE 1. Quantitative polymerase chain reaction (qPCR) was performed for cytochrome B in patients with femur fractures or healthy volunteer controls. Cytochrome B is a specific biomarker for the mitochondrial genome. In this analysis, lower PCR cycle values represent more rapid appearance of a detectable signal and thus higher concentration of DNA template in the specimen. Every cycle doubles the amount of DNA. So a difference of 10 cycles represents a twofold[10] (ie, 1024-fold) increase in starting material and so forth.

Mitochondrial Damage Associated Molecular Patterns Activate Polymorphonuclear $[Ca^{2+}]_i$ Flux

PMNs were stimulated with fracture supernatants at 10% and 20% concentration. Experiments were performed in low-Ca^{2+} environment followed by recalcification of the medium thus visualizing first intracellular Ca^{2+} flux and then Ca^{2+} entry into the cell (Fig. 2). Fracture supernatants caused immediate (t = 30 seconds) Ca^{2+} release by endosomal stores followed by enhanced entry of Ca^{2+} into the cell (t = 150 seconds). F2

Mitochondrial Damage Associated Molecular Patterns Activate Polymorphonuclear P44/42 MAP Kinases

PMNs activated by mitochondria from femur fractures (FFx) (10 μg protein/mL, 10 minutes) were assessed for phosphorylation of p44/42 mitogen-associated protein kinase (MAPK). Total p44/42 and β-actin were used as internal standards. MTD cause brisk phosphorylation of p44/42-MAPK (Fig. 3). Stimulation after treatment with cyclosporine H (CsH, 1 μM, a specific inhibitor of FPR1[7]) completely inhibited activation. Thus, FFx activate PMN P44/42 MAPK through formyl peptide receptors. F3

Mitochondrial Damage Associated Molecular Patterns Cause Polymorphonuclear to Release Matrix Metalloproteinase-9

Excessive matrix metalloproteinase release in inflammation contributes to bystander organ injury through many pathways.[17] We found PMN released matrix metalloproteinase-9 in a dose-dependent fashion when stimulated by MTD from femoral reamings (Fig. 4). Like with p44/42-MAPK, the F4

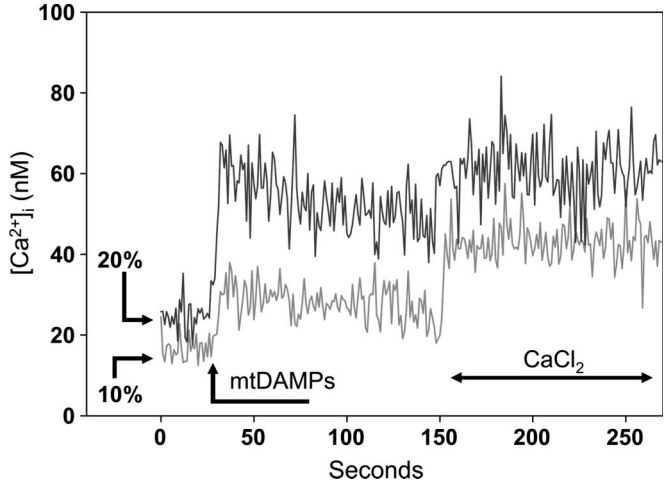

FIGURE 2. Human neutrophils (PMN) were prepared for calcium studies by fura-loading in a cuvette with media low in calcium ($[Ca^{2+}]_o$ approximately 50 nM). We then added either 10% or 20% femur fracture supernatant. An immediate intracellular ''spike'' in $[Ca^{2+}]_i$ is seen. Further ''store-operated'' Ca^{2+} entry is seen on recalcification of the medium at t = 150 seconds. Responses are dose-related. Representative traces are shown; n = 3/condition.

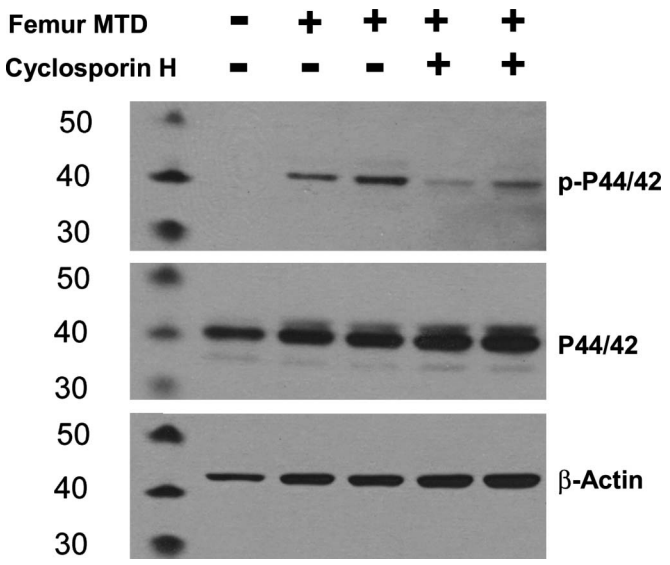

FIGURE 3. Neutrophils were exposed to mitochondrial damage associated molecular patterns (MTD) derived from femur fractures (FFx) (MTD) and assayed by Western blot for the phosphorylation of p44/42 mitogen-associated protein kinase. Damage associated molecular patterns (DAMPs) from FFx rapidly activated this key polymorphonuclear (PMN) kinase. Activation was strongly inhibited by cyclosporine H, an inhibitor of the formyl peptide receptor-1 (FPR1). Total p44/42 and β-actin are shown as controls. $*P < 0.05$ (analysis of variance/Tukey test)

effect was completely reversed by CyH. Matrix metalloproteinase-9release was completely blocked by anti-FPR1 antibodies. These findings show PMN degranulation of matrix metalloproteinases is activated by MTD in FFx through formyl peptide receptors, likely FPR1 (n = 3 replications/condition, $*P < 0.05$ by analysis of variance/Tukey test).

Femur Fracture Mitochondrial Damage Associated Molecular Patterns Cause Polymorphonuclear Interleukin-8 Release

Activated PMN release interleukin-8, which activates PMN and recruits further PMN. Both interleukin-8 and responses to it are increased in ALI/ARDS.[14,15] Here, PMNs were induced to produce interleukin-8 by exposure to MTD from femoral reamings (Fig. 5). The dose response suggests this may occur at the concentrations found in circulating plasma during reaming.

Pulmonary Inflammation in Response to Fracture Mitochondrial Damage Associated Molecular Patterns

MTD from human femoral reaming was injected into rats intravenously. Rats were euthanized 1 hour later. Whole lung homogenates were prepared and assayed for phosphorylation of p44/42 (Fig. 6). We found brisk phosphorylation of p44/42, suggesting FFx MTD cause ingress of activated inflammatory cells into the lung.

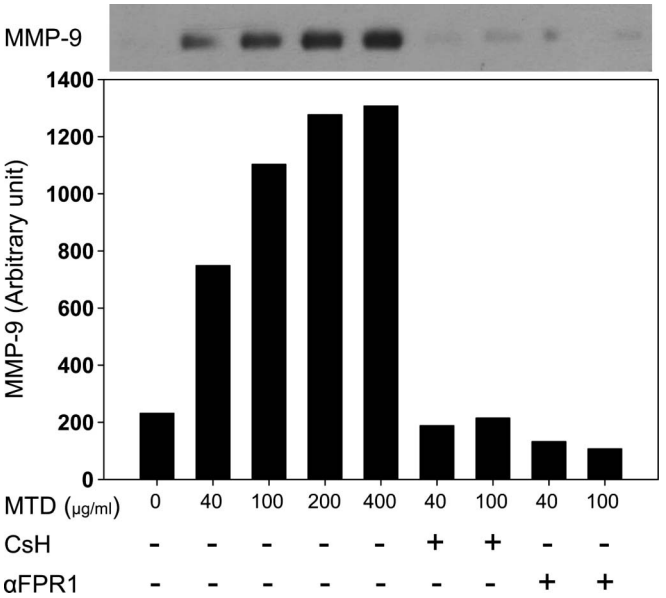

FIGURE 4. Polymorphonuclear (PMN) release matrix metalloproteinase-9 (MMP-9) after exposure to femur fracture (FFx) mitochondrial damage associated molecular patterns (MTD). PMN were exposed to FFx MTD (10 minutes) at the concentrations noted. Supernatants were assayed for MMP-9 by Western blot. MTD caused brisk degranulation of MMP-9 ($*P < 0.05$; analysis of variance/Tukey test). Release was inhibited by cyclosporine H, an inhibitor of formyl peptide receptor-1 (FPR1), or by monoclonal antibodies to FPR1 (αFPR1).

DISCUSSION

Activation of innate immune phagocytic function is required for the clearance of injured tissue that must precede wound repair. What we see here is that the same primary

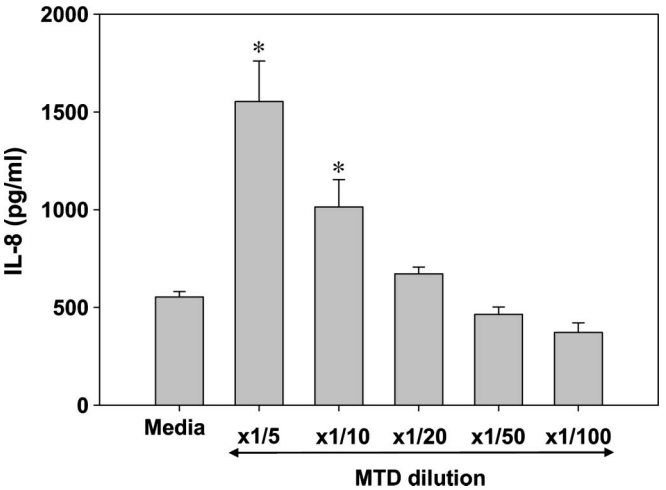

FIGURE 5. Polymorphonuclear (PMN) produce interleukin-8 (IL-8) after exposure to mitochondrial damage associated molecular patterns (MTD) from femur fracture (FFx) reamings. Ten percent and 20% MTD caused brisk release of IL-8 at 4 hours (analysis of variance $P < 0.01$; post hoc Holm/Sidak $P < 0.05$).

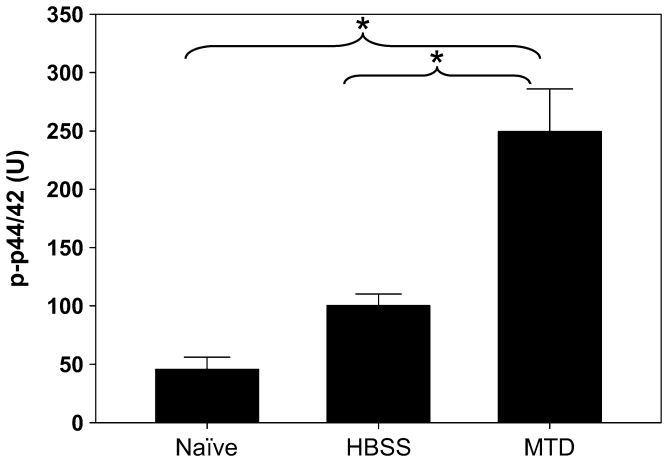

FIGURE 6. Mitochondrial damage associated molecular patterns (MTD) from femur fracture (FFx) reamings were injected into rats intravenously. One hour later, rats were euthanized and whole lung homogenates were assayed for activation of p44/42 by Western blot. We found brisk phosphorylation of p44/42, suggesting the onset of pulmonary inflammation. *P = 0.002 versus naïve (analysis of variance/Holm-Sidak).

mediators initiating local innate immunity can also precipitate systemic activation of innate immunity, clinically manifest as systemic inflammatory response syndrome. We believe systemic inflammatory response syndrome is universal after major fracture/soft tissue injury but that its intensity varies widely. This may reflect factors related to the local wound or reflect variable host response. However, in any case, fracture/soft tissue injury and its management can contribute to clinical systemic inflammatory response syndrome indistinguishable from sepsis without an identifiable infection. Moreover, these manifestations can include ALI or ARDS.

Here, we show femoral fracture wounds (and their repair by reamed nailing) can release mitochondrial debris into the wound and into the systemic circulation. Because mitochondria are evolutionarily derived from bacteria,[10,11] we hypothesized their release by cellular disruption would expose the host to immunologically active "danger signals." Our findings demonstrate that femur fractures and reamed repairs do indeed release mitochondrial DAMPs and that these FFx-derived DAMPs are capable of activating innate immune cells and causing pulmonary inflammation in an animal model.

Our findings support the novel paradigm that fracture/soft tissue injury contribute to the genesis of clinical SIRS by local release of intracellular, mitochondrial-derived DAMPs. Our current studies are limited to evaluation of PMN activation and biochemical studies performed in rats treated with MTD. Moreover, other intracellular motifs can act as "alarmins"[16,18] and such molecules are also likely to participate in local wound repair as well as systemic inflammation. Thus, biologic response modifications based on the effects of MTD alone would be premature and must be approached with caution.

We therefore hypothesize that release of MTD from injured tissues forms a link between tissue trauma and sterile SIRS that can predispose to inflammatory lung injury. More research is required to determine the extent to which release of mitochondrial products by mechanical disruption or other forms of tissue injury is responsible for systemic PMN activation and the evolution of SIRS after clinical injury.

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Mitochondrial Peptides Are Potent Immune Activators That Activate Human Neutrophils Via FPR-1

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Background: Tissue injury from mechanical trauma modulates innate immunity. The resultant systemic inflammatory response syndrome (SIRS) closely mimics clinical sepsis, and bacterial *n*-formyl peptides are septic mediators. Similar formyl peptides exist in mitochondria but little is known about their actions on human neutrophils (PMN).

Methods: Mitochondria were isolated from rat heptocytes and disrupted. Soluble mitochondrial degradation products (MDP) were used to stimulate human PMN. Cytosolic calcium ($[Ca^{2+}]_i$) responses to MDP were assayed with and without antibody blockade of formyl peptide receptor 1 (FPR-1) or formyl peptide receptor like-1 (FPRL-1). Chemotaxis toward MDP was assessed in trans-wells. Oxidative burst to MDP was assessed using carboxy-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate.

Results: MDP caused $[Ca^{2+}]_i$ responses similar to those caused by formyl-Met-Leu-Phe. $[Ca^{2+}]_i$ responses were completely blocked by anti-FPR-1 antibodies, showing activation occurred via the high-affinity, G-protein–coupled FPR-1 receptor. MDP acted on FPR-1 to give chemotactic responses similar to 10 nM formyl-Met-Leu-Phe. MDP also caused dose-dependent oxidative burst.

Conclusion: Formylated mitochondrial proteins are potent immune activators. Acting through the FPR-1 receptor on professional phagocytes, MDP elicits $[Ca^{2+}]_i$ release responses and Ca^{2+} entry via G-protein–coupled pathways. MDP activates chemotaxis and respiratory burst. Our findings suggest a novel paradigm wherein one root cause of SIRS after trauma may be the release of mitochondrial fragments from mechanically damaged tissues. In this paradigm, mitochondrial debris "alarmins" alter host PMN phenotype, activating or suppressing immunity, predisposing to SIRS, sepsis or organ failure.

Key Words: Neutrophils, Trauma, Inflammation, SIRS.

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Systemic inflammatory response syndrome (SIRS) is a well recognized but a poorly understood clinical entity that can occur as a direct result of injury. Its clinical presentation can be indistinguishable from that of infection or sepsis, and SIRS is commonly followed by inflammatory complications that include acute lung injury, adult respiratory distress syndrome, multiple organ dysfunction syndrome (MODS), circulatory failure, and death.[1,2] The prevalence of SIRS is very high, affecting one-third of all inhospital patients, >50% of all intensive care unit (ICU) patients and >80% of surgical ICU patients.[3] MODS occurs in ~28% of patients with SIRS with an associated mortality rate of up to 50%.[4]

Despite a broad consensus as to the clinical existence and definition of noninfectious SIRS, its cause remains elusive and its clinical similarity to bacterial infection frequently complicates the management of ICU patients. It is commonly thought that mechanical trauma activates the immune system when hypoperfusion or other tissue injuries initiate transient dysregulation of cytokine production. This "cytokine storm" then activates innate immune effector cells like neutrophils (PMN), which can damage uninjured (bystander) tissues.[5,6] But strategies blocking receptors for these cytokines have proven of little benefit,[7,8] and there is no clear link between tissue injury per se and cytokine production. Thus, there is a need for further understanding of the events that link mechanical injury to immune modulation and physiologic organ dysfunction if alternative therapeutic modalities are to be developed.

In prokaryotes like bacteria, all protein synthesis begins with the *n*-formyl methionine residue.[9,10] Thus, *n*-formyl methionine peptides such as formyl-Met-Leu-Phe (fMLP) are potent immunocyte activators that activate neutrophils via two specific, G-protein-coupled formyl peptide receptors. These receptors are known as formyl peptide receptor (FPR-1) and formyl peptide receptor like-1 (FPRL-1) and have been extensively studied.[11] Eukaryotic (including human) cytoplasmic proteins do not contain formal peptide residues, but mitochondria closely resemble prokaryotic bacteria in many ways, including the presence of a dedicated circular mitochondrial DNA (mtDNA) genome and initiation of protein synthesis with *n*-formyl methionine.[12] These observations have led to the belief that mitochondria were once free-living saprophytic organisms that became endosymbiotic organelles. Even though at this point in evolution mitochondria only produce 13 endogenous proteins, each one begins with an *n*-formyl methionine residue.[12,13]

We therefore hypothesized that formyl peptides (FP) released when cells are directly disrupted by tissue injury could activate the innate immune system. Specifically, we hypothesized that human neutrophils would express an inflammatory phenotype after exposure to mitochondrial degradation products (MDP), potentially characterized by enhanced calcium mobilization and oxidative burst, and chemotaxis to MDP.

MATERIALS AND METHODS

Reagents and Chemicals

n-Formyl-methionyl-leucyl-phenylalanine (fMLP), ethyleneglycol-*bis*(ß-aminoethylether)-*N*,*N*′-tetraacetic acid

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(EGTA), protease inhibitor cocktail, and dimethyl sulfoxide were purchased from Sigma (St Louis, MO). Fura2-AM, Calcein-AM, and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Molecular Probes (Eugene, OR). Anti-human formyl peptide receptor-1 (FPR-1), anti-FPRL-1, and matrix metalloproteinase 2 (MMP2) antibodies were purchased from R&D (Minneapolis, MN). Hanks' Balanced Salt Solution and phosphate-buffered saline were purchased from (GIBCO; Invitrogen, Grand Island, NY).

Mitochondrial Isolation

Preparation of MDP

Mitochondria were isolated from rat liver acquired from male Sprague-Dawley rats (300–350 g, Charles River). Rat liver was morcellated, and then processed as per the Mitochondria Isolation Kit for tissue (PIERCE, Rockford, IL) using dounce homogenization per the soft tissue protocol supplied by the manufacturer. Mitochondria were isolated under sterile conditions at 4°C. Isolated mitochondrial pellets were stored on ice pending further processing. Isolated mitochondrial pellets from 200 mg tissue specimens were suspended in 1 mL of buffer containing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10 mmol/L glucose, 20 mmol/L, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). Hanks Balanced Salt Solution (HBSS) was used for chemotaxis assays. Protease inhibitor cocktail (1:100) was added to the suspension, which was then subjected to sonication on ice using VCX130-Vibra Cell (Sonics and Materials, Newtown, CT) at 100% amplitude, three times for 30 seconds each. The disrupted mitochondrial suspension was then centrifuged at 12,000*g* for 10 minutes at 4°C. The supernatant containing soluble MDP was removed and stored at −20°C for experiments after readdition of protease inhibitors (1:100). The dilution was standardized such that the calcium release response of human PMN was similar at 1/100 dilution to the response to 1 nM fMLP. Protein concentration was determined by BCA assay (PIERCE, Rockford, IL) and in general was in the range of 1.2 mg protein/mL. MDP samples are routinely assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis gel electrophoresis and by qPCR of their DNA content to exclude the presence of cytosolic or nuclear debris.

Neutrophil Isolation

A detailed protocol is described elsewhere.[14] Briefly, human whole blood from healthy volunteers was collected in heparinized (10 U/mL) 15 mL tubes (Becton, Dickinson, NJ), by venipuncture and centrifuged for 10 minutes at 1,000 rpm. The platelet-rich plasma was removed. The buffy coat and upper 2 cm of red blood cells were then centrifuged at 1,500 rpm for 30 minutes after layering onto 5 mL Polymorphoprep gradient solution (Robbins Scientific, Sunnyvale, CA). The supernatant containing the mononuclear cell layer was now aspirated and discarded. Neutrophil layers were now collected, diluted with an equal volume of 0.45% NaCl solution, and allowed to rest for 5 minutes at room temperature to restore normal osmolarity. PMN were then washed with RPMI 1:10 (Mediatech, Herndon, VA) and centrifuged at 1,000 rpm for 10 minutes. Hypotonic lysis was performed on ice to remove any contaminating red blood cells. This method results in a preparation containing ≥98% neutrophils by stain and with a viability of ≥99% as assessed by Trypan Blue exclusion. Neutrophil pellets were now resuspended in HBSS with 5% fetal bovine serum (FBS) for chemotaxis assays or in HEPES (hydroxyethylpiperazine ethanesulfonic acid) buffer solution (140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10 mmol/L glucose, and 20 mmol/L HEPES) with 0.1% bovine serum albumin for calcium and oxidative burst experiments.

Calcium Dye Loading

PMN were incubated in 2 μmol/L fura-2-acetoxymethyl ester (fura-2AM; Molecular Probes Inc., Eugene, OR) for 30 minutes at 37°C in dark. Cells were then divided into 200 μL aliquots and put on ice. Individual aliquots were rewarmed to 37°C before each experiment and then centrifuged at 5,000 rpm for 30 seconds in a microcentrifuge. The supernatant was discarded, and the cells were resuspended in 200 μL HEPES buffer solution with 0.1% bovine serum albumin before loading to the cuvette containing 2.8 mL of the same buffer. All experiments were begun in "nominally calcium-free" media conditions created by adding 0.3 mmol/L EGTA to cuvettes before loading the cells. Extracellular calcium was then added as indicated.

Spectrofluorometry

PMN cytosolic calcium ($[Ca^{2+}]_i$) was measured by recording fura-2AM fluorescence at 505 nm, using 340/380 nm excitation in a Fluoromax-2 spectrofluorometer (Jobin-Spex, Edison, NJ) with constant stirring at 37°C. PMN were permeabilized by 100 μmol/L digitonin (Molecular Probes) to measure R_{MAX} followed by adding 15 mmol/L EGTA solution to measure R_{MIN} at the end of individual experiments to achieve optimal calibration. The fluorescence of a sample cell suspension treated with 100 μmol/L digitonin and 2 mmol/L $MnCl_2$ was subtracted from total fluorescence. $[Ca^{2+}]_i$ was then calculated from the 340/380 nm fluorescence ratio using our modifications of the methods of Grynkiewicz et al.[15]

Chemotaxis

PMN chemotaxis was studied using 96-well Multiscreen-MIC plates (Millipore; Billerica, MA). The bottom of each upper well is made of a polycarbonate membrane with 3 μm pores. After isolation, PMN were incubated in calcein-acetoxymethyl ester, 3 μg/mL (calcein-AM, Molecular Probes) for 30 minutes at 37°C in dark.[16] Pellets were then resuspended in HBSS with 5% FBS. Final PMN concentrations were adjusted to 1×10^7 cells/mL. For each experiment, the upper well contained 100 μL PMN suspension (10^6 calcein-loaded PMN per well) and the lower well contained 150 μL of putative chemoattractant diluted in Hanks' balanced salt solution with 5% FBS. "Blanks" without chemoattractant were used to determine the random migration (chemokinesis) of PMN plated in the absence of chemoattractant in the lower well. The entire system was now incubated for 50 minutes at 37°C in the dark. The insert wells were now carefully removed. PMN aliquots from each lower

chamber were transferred to a 96-well, U-bottom plate for counting. We set aside 10^6 fura-loaded cells to create a standard curve of serially diluted PMN. Chemotaxis was measured as the fluorescence of PMN entering the lower well as measured at an excitation and emission of 494/517 nm. Control experiments were also performed in the presence of monoclonal blocking antibodies to FPR-1, isotype control antibodies to FPRL-1, and MMP2 or in the presence of the FPR-1 inhibitor Cyclosporin H[17] (CsH, 10 μmol/L).

Oxidative Burst

Isolated PMN were resuspended in HEPES buffer solution containing 0.9 mmol/L $CaCl_2$. The final concentration was 2×10^7 cells/mL. The cells were then incubated with 10 μmol/L (H_2DCFDA; Molecular Probes) for 30 minutes at 37°C in dark. The cells were not washed to measure the total (i.e., intracellular plus extracellular) production of oxidants. Dye-loaded cell suspensions (50 μL/well) were added to 96-well, U-bottom plates containing 50 μL/well of varying concentrations of MDP, or a positive control. HEPES buffer with 0.9 mmol/L Ca^{2+} was used as a negative control. Oxidative burst was assessed by recording H_2DCFDA fluorescence at an excitation/emission of 495/527 nm at 37°C for 100 minutes for time course or at time 0 minutes and 30 minutes for assessing concentration dependence.

Statistical Analysis

All $[Ca^{2+}]_i$ transient results reported are measured as the mean (±SEM) change from basal $[Ca^{2+}]_i$ in nanomoles (nM) per liter. Study data were assessed for statistical significance using analysis of variance with post hoc testing or *t* tests where appropriate. All data are reported as the mean ± SEM, and statistical significance was accepted at a p value <0.05.

RESULTS

MDP Causes PMN Ca^{2+} Mobilization Responses Similar to fMLP

Fura-loaded human PMN were treated with MDP. G-protein–coupled calcium depletion from endoplasmic reticulum stores and subsequent calcium influx were temporally separated by stimulating the cells under nominally calcium-free conditions and restoring extracellular calcium concentration after 130 seconds. Brisk, dose-dependent Ca^{2+} depletion responses were noted (Fig. 1). After our initial dose response experiments, we created stock solutions where ~1/100 dilution of the MDP (1.2 μg protein/mL) yielded a spike in PMN intracellular calcium concentration ($[Ca^{2+}]_i$) of 110 nM to 120 nM. In our experience, this is approximately the same calcium depletion as is caused by 1 nM fMLP (Fig. 2, A) and about twice the response seen with 1 pM fMLP (Fig. 2, B). On recalcifying the media, the Ca^{2+} influx observed was usually indistinguishable from the leak current typically seen on recalcifying nonstimulated cells.

MDP Activates PMN Ca^{2+} Mobilization via FPR-1

Mitochondrial proteins are *n*-formylated and PMN express two *n*-formyl peptide receptors; the high-affinity FPR-1 (K_D, ~ 5 nM) and the low-affinity FPRL-1 (K_D, 1–2 μmol/L). To assess the dependency of PMN calcium mobilization responses to MDP on formylated mitochondrial proteins, we pretreated PMN with blocking antibodies to the formyl peptide receptors. Cells were incubated with or without anti-FPR-1 or anti-FPRL-1 antibody for 10 minutes at 37°C. Antibodies to FPR-1 completely blocked Ca^{2+} mobilization by MDP whereas antibodies to FPRL-1 had no effect (Fig. 2, A). In control studies, anti-FPR antibodies demonstrated complete inhibition of responses to fMLP (Fig. 2, B). Antibodies to FPRL-1 (Fig. 2, C) and isotype control antibodies (to MMP2, data not shown) showed no nonspecific inhibition of MDP effect.

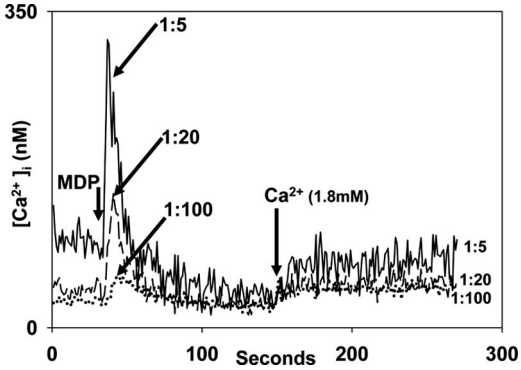

Figure 1. Typical dose-dependent Ca^{2+} release responses to MDP. This MDP preparation yields a spike of over 300 nM at a 1:5 dilution. Progressive dilutions yield attenuated Ca^{2+} release responses. Only very high concentrations of MDP (1:5 dilution) cause any detectable increase in calcium influx on re-calcifying the medium at t = 150 seconds.

MDP Is a Potent PMN Chemoattractant

PMN chemotaxis to MDP was studied in a trans-well system. When varying concentrations of MDP were applied to the trans-wells maximal chemotactic responses were observed at a 10-fold dilution of the original MDP preparation (Fig. 3, *upper solid line*). The bell-shaped chemotaxis curve seen is characteristic of PMN responses to fMLP: as with other GPC chemoattractants, very high agonist concentrations lead to homologous receptor down-regulation. In addition, the maximal chemotactic response was seen at a 10-fold dilution of MDP (i.e., approximately the equivalent of 10 nM fMLP), which is consistent with the maximal chemotactic responses to fMLP that we have seen in previous studies.[16]

PMN Chemotaxis to MDP Depends on the FPR-1 Receptor

To assess the dependency of PMN chemotaxis to MDP on formylated proteins, we again pretreated PMN with blocking antibodies to FPR-1 receptors (Fig. 3, *lower dashed line*). Chemotactic responses to optimal doses of MDP were strongly inhibited ($p < 0.006$; Fig. 3; $p < 0.05$ analysis of variance/Holm-Sidak), demonstrating the involvement of FPR-1. Control experiments also demonstrated complete inhibition of chemotaxis to 10 nM fMLP (Fig. 3, *Panel 2* and

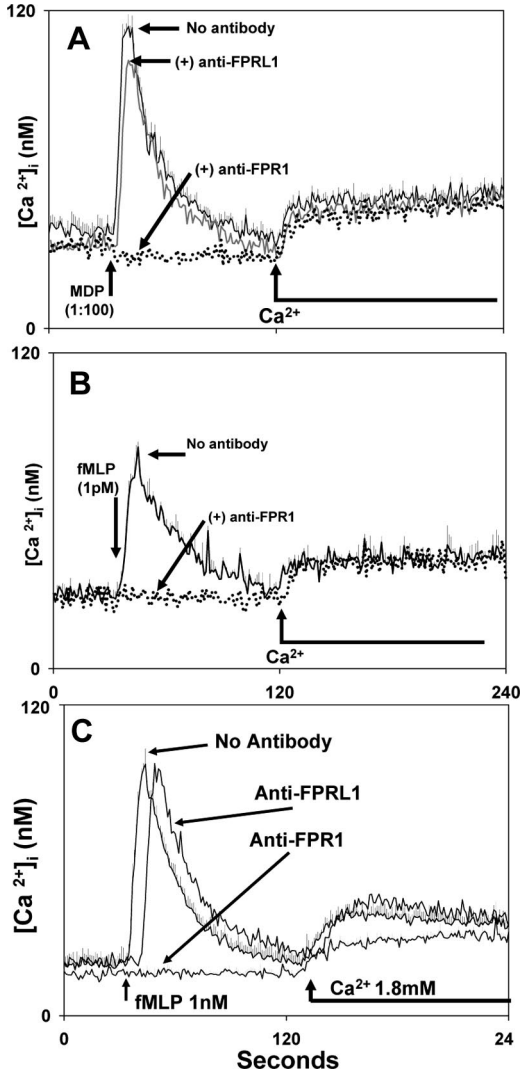

Figure 2. In the upper panel (*A*), we see that without prior antibody treatment human PMN stimulated with MDP demonstrate a brisk calcium release from endosomal stores. After incubation with anti-FPR-1 (10 minutes, 37°C), calcium release is completely blocked. Antibody to FPRL-1 has no effect. In the lower panels, we see control experiments using the same cells stimulated by fMLP. Antibodies to FPR-1 quantitatively block Ca^{2+} store release responses to 1 pM (*B*) to 1 pM fMLP (*B*) where antibodies to FPRL-1 have no effect (*C*). All experiments are n = 3–4. Baseline conditions are shown as solid lines with SE bars.

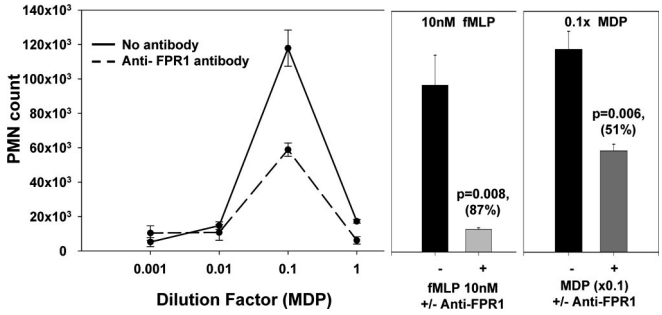

Figure 3. Dose-response curves for chemotaxis toward rat hepatocyte MDP in untreated (upper trace; n = 3) and anti-FPR-1 antibody treated (lower trace; n = 3) PMN. Chemotaxis was suppressed over a broad range of MDP doses using FPR-1 blockade. MDP protein concentration (×1) =1.23 mg/mL, * $p < 0.05$ by Student's *t* test.

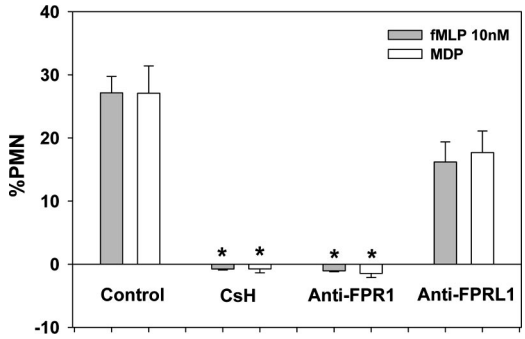

Figure 4. PMN chemotaxis to both MDP and fMLP are noted to be suppressed both by blocking antibodies to FPR-1 and by the FPR-1 inhibitor cyclosporin H (CsH). PMN chemotaxis was not suppressed by blocking antibodies FPRL-1 or isotype control antibodies (to MMP2, not shown).

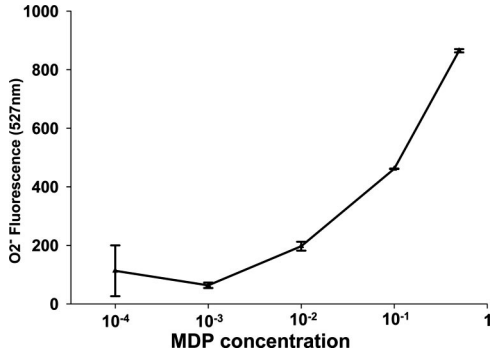

Figure 5. MDP causes PMN respiratory burst in a dose dependent manner. Total respiratory burst was measured as absolute H_2DCFDA fluorescence units (excitation/emission 495/527 nm). MDP protein concentration (×1) =1.23 mg/mL.

Fig. 4) and also that anti-FPR-1 was equally effective against fMLP and MDP. Further control experiments showed that antibodies to FPRL-1 were ineffective against both MDP- and fMLP-induced chemotaxis (Fig. 4). Isotype control antibodies to MMP2 (not shown) had no effect on chemotaxis of MDP or fMLP but chemotaxis to each was markedly inhibited by the FPR-1 inhibitor Cyclosporin H[17] (Fig. 4).

MDP Causes PMN to Undergo Oxidative Burst

MDP was found to activate PMN respiratory burst as assessed by H_2DCFDA fluorescence after 30 minutes of coincubation. Varying concentrations of MDP were used with dilution factors of 0.001–0.5, and these experiments clearly demonstrated dose-dependent free-radical generation (Fig. 5).

DISCUSSION

Activation of innate immunity is a key step in early host responses to invasive infection. It is also critical to the phagocytosis and clearance of mechanically injured tissue and thus to the initiation of tissue repair. Both processes are required for host survival, but whether initiated by injury or infection, innate immune activation initiates the inflammatory process we recognize clinically as SIRS. In turn, SIRS can predispose to vital organ injury and contribute to the morbidity and mortality seen in major trauma patients. The prevalence of SIRS is exceedingly high and has been reported in up to 29% of all trauma admissions.[18]

Patients suffering tissue injury without an identifiable source of infection often manifest clinical characteristics that are virtually indistinguishable from sepsis. By consensus, such patients are said to have noninfectious SIRS, and this group of patients has a high rate of MODS and MODS-associated mortality.[4] The molecular events linking trauma to SIRS remain poorly understood however, and without understanding the molecular etiology of noninfectious SIRS, it remains difficult to distinguish infectious SIRS from noninfectious causes. Thus, without such a mechanistic understanding, we will continue to treat patients with unnecessary and potentially deleterious antimicrobials while failing to identify appropriate molecular targets for the therapy of their SIRS.

Prokaryotic protein synthesis is unique in that the initiating amino acid is always *n*-formyl methionine.[9,10] Bacterial peptides retaining this formyl group after translation are called FP. In 1975, Schiffmann et al.[19] showed that bacterial-derived FP are potent PMN chemoattractants and that the formyl group is essential for this activity. Since then, formylated peptides and their synthetic prototype fMLP have been extensively studied for their role in activation of immunity and host defenses. Mammalian mitochondria (like prokaryotes) require f-Met-t-RNA for initiation of protein synthesis and mitochondrial DNA encoded peptides begin with *n*-formyl-methionine. It seemed plausible to us that disruption of mammalian cells would release mitochondrial products capable of activating PMN in a manner similar to bacterial or synthetic FP.

The current studies address those issues. They show that MDP mobilize cell calcium flux via FPR-1, thus causing PMN to express an inflammatory phenotype in the absence of other stimuli. This phenotype includes increased ($[Ca^{2+}]_i$) levels activation of chemotaxis and the stimulation of respiratory burst.

Tissue injury is known to release mitochondrial peptides into the circulation in the absence of bacterial infection.[20] Moreover, PMN formyl peptide receptors can be upregulated by trauma.[21] Because direct destruction of soft tissues is a *sine qua non* of major blunt force injury, we hypothesize that release of MDP from injured tissue may be the crucial link between tissue trauma and a PMN-mediated sterile inflammatory state. We are therefore currently working to determine the extent to which release of mitochondrial products by mechanical tissue injury is responsible for systemic activation of PMN, and the development of SIRS after human, clinical injury.

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DISCUSSION

Dr. Ronald V. Maier (Seattle, Washington): Thank you very much. I thank the audience for staying. And although we say this a lot I really would like to congratulate the

authors on a very unique concept that they're beginning to investigate.

I think it's really going to become increasingly important as it is recognized, the impact it has on patients and our potential to use this information to treat our patients.

We've had in this field for a long time this sort of enigma that it appears like formylated peptides from bacteria were the reason why we got sick from sepsis.

But, as was mentioned in the introduction, many patients that have become injured become septic-like with SIRS or the sepsis syndrome and we could never figure out what the stimulus was so we made up excuses like there was bacterial translocation from the gut which you can show in mice and has really never been shown in humans.

And we just sort of said, well, you can't find it but it's there because that's why they're getting this sepsis syndrome.

And Polly Matzinger?? first started pushing this paradigm that when you injure tissue you release molecules that are recognized, similar to the molecules that come out of the septic response and that drives this systemic response that causes systemic disease and multiple organ failure.

And this has become increasingly recognized. These products from damage are recognized by the same receptors like the toll-like receptors.

And now we're showing that another receptor that recognizes formylated peptides recognizes peptides that come out of our own endogenous bacterium that lives in every cell which is the mitochondrial. So I think it's critical studies and I think it's been, this study shows the beginning of that evolution.

My questions for the authors are primarily on their hypothesis and that is although there is some proof that when you injure a tissue – like if you take a mouse and you mash its liver in half you can actually measure some mitochondrial damage products in the circulation – what data do you have showing that after a significant injury there are circulating mitochondrial products in humans?

And, to what degree do those correlate with the severity of injury in predicting of MOF?

Secondly, in sort of correlation with that is in your own studies does it take total cell disruption to release these products or is it also partially an active process like with HMGB-1 and other molecules that the cells can actually secrete these products without having to have total cell death?

The second question is, after the neutrophils see these products do they become paralyzed? Does it lead to immuno paralysis? And have you looked at these cells after they've been exposed? And are they effete or can they still respond as innate immune cells?

This would be similar to the, also the issue as to whether these mitochondrial peptides can induce apoptosis. And do you know if these byproducts of injury can induce apoptosis as a mitochondrial source?

Third, the PMN response, as Dr. Hauser knows, is usually very brief and its relevance to multiple organ failure is still debated. So the question I have is as you mention many innate cells have this receptor, what's the effective of these peptides on stimulating monocytes and macrophages which, as most of us know, are much more critical cells in this response than the neutrophil?

And then, lastly, and you can tell me you just don't know but why do mitochondria produce formylated peptides? Why do bacterial produce formylated peptides?

What evolutionary benefit does formulating a peptide give you? And if it gives you a benefit, why don't humans do it? Thank you.

Dr. Yasuhide Inoue (Japan): My name is Inoue from Saiseikai Fukuoka General Hospital, Emergency and Critical Care Medical Center, Japan.

I thank you for your excellent data and nice presentation.

I have two questions about your experiment. Question 1, you chose liver and muscle cells to isolate mitochondria so how did you choose that kind of donor organs to isolate mitochondria?

And, Question 2, you showed activation and migration change of neutrophils. How about ATP release or neutrophil elastase stimulation?

Again, thank you for nice presentation.

Dr. Mustafa Raoof (Boston ,Massachusetts): to answer Dr. Maier's first question as to whether this is relevant in humans, do we actually see evidence that injury will lead to mitochondrial debris in the circulation. I have an extra slide that I can share with the audience. This slide shows a real-time PCR experiment assaying mito chondrial DNA in the peripheral circulation. Trauma patients with an ISS score greater than 25 are in red and controls are in black. Mitochondrial DNA is clearly elevated in the peripheral circulation in trauma patients. This suggest trauma patients also have increased mito chondrial proteins in the circulation.

To suggest whether mitochondrial debris in the peripheral circulation are important we have begun looking at lung homogenates from rats injected with mito chondrial debris. Our preliminary findings suggest that mitochondrial debris definitely increased neutrophil activity (measures as MMP-8 accumulation) in the lung tissues. This could suggest a mechanism for the genesis or ARDS and organ failure.

The other question Dr. Maier raised was whether mitochondrial debris could cause apoptosis. We haven't looked at that yet, but it's a good idea and a question that we should look at in the future.

Does mitochondrial debris activate monocytes and macrophages? We're still working on what happens to neutrophils. But we know that monocytes have FP receptors, so my guess is they would be (activated). But unless we look at it, we'll never know.

What evolutionary advantages do bacteria gain by having formylated peptides? I'm not sure what the evolutionary advantage would be but we know that most formylated peptides get deformylated in bacteria but mitochondria do not express formylase like bacteria do. There are certain differences between bacteria and mito chondria in terms of the quantity of formylated peptides they produce. Bacteria produce formylated peptides but they are deformylated (by deformylase). We don't know why they do that. Certainly, these and other differences can be looked into and may point

us to the significance of formylgroups in formyl peptides. As I suggested earlier, this is preliminary data and this just opens a brand-new field of research for us.

Dr. Inoue's questions was about liver and muscle mitochondria and how we chose them. For these preliminary studies we chose to isolate liver and muscle mitochondria from naïve rats because of convenience. Subsequently, we have moved on to testing human muscle mito chondrial extracts on human neutrophils. We also now use rat muscle mitochondrial proteins to stimulate rat PMN in vivo.

Answering the last question by Dr. Inoue, we looked at the possibility of ATP in mitochondrial debris might activate PMN calcium release through adenosine receptors. So we treated the mitochondrial debris preparation with apyrase (ATPase). Preparations treated with Apyrase showed complete inhibition of calcium spikes in response to ATP but apyrase did not change the calcium release response of neutrophils to mitochondrial debris. So the calcium response is not because of ATP, and is in fact seems caused by formyl peptides in mitochondrial debris.

Thank you.

Shock: Injury, Inflammation, and Sepsis: Laboratory and Clinical Approaches

Plasma Bacterial and Mitochondrial DNA Distinguish Bacterial Sepsis from Sterile SIRS and Quantify Inflammatory Tissue Injury in Nonhuman Primates

--Manuscript Draft--

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Abstract:	Systemic inflammatory response syndrome (SIRS) is a fundamental host response common to bacterial infection and sterile tissue injury. SIRS can cause organ dysfunction and death but its mechanisms are incompletely understood. Moreover, SIRS can progress to organ failure or death despite being sterile or after control of the inciting infection. Biomarkers discriminating between sepsis, sterile SIRS and post-infective SIRS would therefore help direct care. Circulating mitochondrial DNA (mtDNA) is a damage-associated molecular pattern (DAMP) reflecting cellular injury. Circulating bacterial 16S-DNA (bDNA) is a pathogen-associated pattern (PAMP) reflecting ongoing infection. We developed qPCR assays to quantify these markers and predicted their plasma levels might help distinguish sterile injury from infection. To study these events in primates we assayed banked serum from papio baboons that had undergone a brief challenge of intravenous Bacillus anthracis deltaSterne (modified to remove toxins) followed by antibiotics (anthrax) that causes organ failure and death. To investigate the progression of sepsis to "severe" sepsis and death we studied animals where anthrax was pretreated with drotrecogin alfa (aPC), which attenuates sepsis in baboons. We also contrasted lethal anthrax bacteremia against non-lethal E.coli bacteremia and against sterile tissue injury from Shiga-like toxin-1 (Stx1). bDNA and mtDNA levels in timed samples were correlated with blood culture

	results and assays of organ function. Sterile injury by Stx1 increased mtDNA but bDNA was undetectable: consistent with the absence of infection. The bacterial challenges caused parallel early bDNA and mtDNA increases, but bDNA detected pathogens even after bacteria were undetectable by culture. Sub-lethal E.coli challenge only caused transient rises in mtDNA consistent with a self-limited injury. In lethal anthrax challenge (n=4) bDNA increased transiently but mtDNA levels remained elevated until death, consistent with persistent septic tissue damage after bacterial clearance. Critically, aPC pre-treatment (n=4) allowed mtDNA levels to decay after bacterial clearance with sparing of organ function and survival. In summary, host tissue injury correlates with mtDNA whether infective or sterile. mtDNA and bDNA PCRs can quantify tissue injury incurred by septic or sterile mechanisms and suggest the source of SIRS of unknown origin.
Response to Reviewers:	To the Referees: Thank you for your patience, we apologize for any missed communications. We certainly felt we had responded fully before, but accept that we must have simply 'missed the boat'. We have now gone through the concerns in assiduous detail and hope that we will have made up for any prior (unintended) lack of responsiveness. Reviewer #3: QUERY: I am disappointed in the response by this esteemed group. The reviewers had great suggestions and comments and the confusions about the activated protein c persists. The responses of the authors are somewhat disrespectful and I highly recommend to clarify the various concerns raised by the reviewers and incorporate them into the manuscript. I was asking the authors whether there is a difference between gram negative gram positive and fungal infections in your responses. RESPONSE: We thank you for that esteem and are truly sorry if we have engendered any disappointment. We certainly tried to respond fully and there was never any disrespect meant. We did not seek to be evasive in any way or to disregard any questions. Perhaps we misunderstood some of the points that were being made or simply thought things were obvious to others that were not. But if we failed to respond adequately it certainly wasn't for lack of trying. In any case, we hope to remedy any misunderstandings. Taking the second query first (since it is simpler) we didn't try to compare gram negative and gram-positive sepsis, but find no real differences between gram-negative and gram-positive infection using these methods. Our methods identify Gram-positive and Gram-negative bDNA with identical sensitivity (Supplementary Figure 1) and our methods isolate DNA efficiently from both GPC and GNR. Fungal sepsis is quite a different story: since fungi have mitochondria our methods would not be applicable to fungal infections. Perhaps these points were not clarified, but we have now specified them in the methods (P7, 'rtPCR Protocols', para1). We sincerely hope that was the issue the referee wanted us to address. With respect to using aPC samples as part of the primary design of the study, we started out trying to address a general question that applies equally to gram positive and negative infection: ie, do endogenous stimuli or residual bacterial stimuli cause the continued SIRS and lethality seen after bacteremia in some cases while allowing dissipation of SIRS and survival after the resolution of bacteremia in other cases. The observation that aPC rescues baboons lethal anthrax sepsis after apparent sterilization was our starting point. Thus our premise was that baboon anthrax with and without "aPC rescue" offered a unique opportunity to examine the mechanisms of "endogenous SIRS" since identical bacterial insults led to such radically different outcomes. Although we thought this was clear in the original manuscript and further clarified in the revision we have now expounded on this premise even further in multiple locations in the re-written manuscript (Abstract, Introduction P5, Para2, P6 Para2, Results P10, Para1, Discussion P20-21.) We specify this model has two major strengths: 1) that it examines responses to sepsis directly in a primate model and 2) that the bacterial stimuli used are identical. We accept that 1) the mechanisms of aPC action in baboon anthrax are incompletely understood and 2) aPC appears more effective in baboon models than it has proven to be in clinical trials. These pros and cons of this model are now discussed in detail [P5 Para2; P6 Para2; P18 Para4; P20 Para3]. Precisely because "aPC rescue" was a single model with those limitations we studied non-lethal E. coli infusion and Stx1 infusion to compare the production of DAMPs in self-limited

bacteremia and toxic tissue injury as a further models and allowing us to make generalizable statements about generation of DAMPs in SIRS and sepsis. We hope this is now crystal-clear.
We should point out (going back to the Referee's query about Gram-positive and Gram-negative infections) that anthrax is a Gram-positive organism where E. coli is gram negative. Both caused mtDNA release during IV infusion, but this study does not really allow us to comment on differences in "Danger" response to Gram-positive and Gram-negative organisms. As with progression to lethality, we think that addressing that issue generically would require evaluation of more than one pathogen model in each group. So where that is a fascinating question, it should be addressed in a dedicated study. Also as we note above, studying DAMP release in fungal infections will require different tools.
Thus each model we used has limitations: that is why we studied sera from multiple models allowing us to quantify DAMPs and PAMPs in 1) sterile tissue injury, 2) self-limited bacteremia, 3) bacteremia that progresses to death due to SIRS after sterilization and 4) identical bacteremia where SIRS and death are prevented by aPC rescue. We believe that this combination of methods yielded important, generalizable results. We accept all of the referees' concerns as to the limitation of the individual methods however, and hope that they have now been adequately addressed. Most important, we now discuss all these concerns in detail in the introduction, methods and discussion and believe this thorough discussion allows the reader to evaluate the data presented in a more complete context.

Reviewer #4:
QUERY: This is a revised submission. There was originally a high level of enthusiasm for these studies. However, there were a number of methodological and theoretical concerns that reduced significantly enthusiasm. The authors have responded to the criticisms by primarily arguing them away, or addressing them in the rebuttal. Two of the reviewers recommended removing the aPC studies, but the authors decided otherwise. With that said, the authors are entitled to write the paper their way, and this reviewer is willing to yield on these issues.

RESPONSE: We thank the referee for that original enthusiasm and hope to regain it. The aPC-rescue data is central to our original hypothesis and therefore how we structured and interpreted the data as a whole. We do understand the need to address all methodologic and theoretic concerns as to the use of the aPC model although we did not interpret the other referees' critiques as requesting the removal of the aPC studies. Rather, we saw them as asking for explanations of how we used and interpreted this data. We thought we had addressed those concern in the first revision, but clearly we did so inadequately. We have addressed those issues extensively now both in the referee responses and the manuscript. We would ask that the referee read the very detailed responses to Referee 3. Most important, we now discuss the strengths and limitations of our approach exhaustively in the manuscript [P5 Para2; P6 Para2; P18 Para4; P20 Para3] and in no way simply rebut the referees' critiques.

QUERY: ….there remains one point that needs to be addressed for scientific accuracy and complete transparency.
1. It must be clearly stated in the Methods that this is a retrospective analysis of samples obtained from an ongoing study for other purposes. This is what the authors wrote in their response to the reviewers:
"The experiments were not performed expressly to evaluate mtDNA and bDNA but are part of larger on-going collaborative immunology studies. Detailed methods are provided because the aPC studies have not yet been published and we felt the need to communicate the mtDNA/bDNA observations in a timely manner took precedence over other considerations. It takes years to do these nonhuman primate studies. Hundreds of samples from each experiment are stored routinely and used for many purposes with many collaborators."
Something to this effect must be included in the methods to clarify that these studies were performed over a period of time in the past for other purposes, and these samples were analyzed after the fact for the purpose of these studies.

RESPONSE:
Again, we thought that was clarified in the first revision but apologize if the changes

	were inadequate. We have now included the requested language in the METHODS under “Animal Procedures” as well as emphasized this point more widely throughout the manuscript by referring to ‘archived’ or ‘stored’ samples eg in the Introduction P5, Para 2. Once more, we thank the referees for their thorough review and hope that all the points raised have now been fully addressed.

September 16, 2012

Dear Dr. Chaudry / Irshad:

This letter accompanies our second re-submission of the manuscript "Plasma Bacterial and Mitochondrial DNA Distinguish Bacterial Sepsis from Sterile SIRS and Quantify Inflammatory Tissue Injury in Nonhuman Primates" by Tolga Sursal, Deborah Stearns-Kurosawa, Kiyoshi Itagak, Sun-Young Oh, Shiqin Sun, Shinichiro Kurosawa and Carl J. Hauser.

We again thank the referees for their comments. We appreciate the reviewers' additional suggestions and have diligently addressed their comments in detail.

We have highlighted changes to the manuscript text in red. These changes are quite extensive so in some cases we will simply make reference to the entire section in the text that encompasses the response to the referee. In all cases however, we have thoroughly considered and directly responded to every criticism. We apologize for any prior missed communications and hope that the referees will find the responses appropriate and complete

Yours truly,

Carl

Carl J. Hauser MD, FACS, FCCM.

Plasma Bacterial and Mitochondrial DNA Distinguish Bacterial Sepsis from Sterile SIRS and Quantify Inflammatory Tissue Injury in Nonhuman Primates

Tolga Sursal[1*], Deborah J Stearns-Kurosawa[2*], Kiyoshi Itagaki[1], Sun-Young Oh[2**], Shiqin Sun[1**], Shinichiro Kurosawa[2‡], Carl J Hauser[1‡]

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Running Head: Distinguishing bacterial and sterile SIRS

Keywords: Sepsis, Inflammation, Bacteremia, Systemic Inflammatory Response Syndrome, Trauma

Conflicts of Interest: No conflicts of interest for any authors

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Abstract

Systemic inflammatory response syndrome (SIRS) is a fundamental host response common to bacterial infection and sterile tissue injury. SIRS can cause organ dysfunction and death but its mechanisms are incompletely understood. Moreover, SIRS can progress to organ failure or death despite being sterile or after control of the inciting infection. Biomarkers discriminating between sepsis, sterile SIRS and post-infective SIRS would therefore help direct care. Circulating mitochondrial DNA (mtDNA) is a damage-associated molecular pattern (DAMP) reflecting cellular injury. Circulating bacterial 16S-DNA (bDNA) is a pathogen-associated pattern (PAMP) reflecting ongoing infection. We developed qPCR assays to quantify these markers and predicted their plasma levels might help distinguish sterile injury from infection. To study these events in primates we assayed banked serum from *papio* baboons that had undergone a brief challenge of intravenous *Bacillus anthracis* deltaSterne (modified to remove toxins) followed by antibiotics (anthrax) that causes organ failure and death. To investigate the progression of sepsis to "severe" sepsis and death we studied animals where anthrax was pretreated with drotrecogin alfa (aPC), which attenuates sepsis in baboons. We also contrasted lethal anthrax bacteremia against non-lethal *E.coli* bacteremia and against sterile tissue injury from Shiga-like toxin-1 (Stx1). bDNA and mtDNA levels in timed samples were correlated with blood culture results and assays of organ function. Sterile injury by Stx1 increased mtDNA but bDNA was undetectable: consistent with the absence of infection. The bacterial challenges caused parallel early bDNA and mtDNA increases, but bDNA detected pathogens even after bacteria were undetectable by

culture. Sub-lethal *E.coli* challenge only caused transient rises in mtDNA consistent with a self-limited injury. In lethal anthrax challenge (n=4) bDNA increased transiently but mtDNA levels remained elevated until death, consistent with persistent septic tissue damage after bacterial clearance. Critically, aPC pre-treatment (n=4) allowed mtDNA levels to decay after bacterial clearance with sparing of organ function and survival. In summary, host tissue injury correlates with mtDNA whether infective or sterile. mtDNA and bDNA PCRs can quantify tissue injury incurred by septic or sterile mechanisms and suggest the source of SIRS of unknown origin.

Introduction

The systemic inflammatory response syndrome (SIRS) can occur either in the setting of sepsis due to pathogens or in a wide variety of circumstances where sterile processes activate inflammation. Both types of initiating events signal “danger” to the immune system. In terms of molecular pathogenesis however, sterile SIRS reflects activation of innate immune pathways by host damage-associated molecular patterns (DAMPs) (1), whereas sepsis is initiated by pathogen-associated molecular patterns (PAMPs) originating from infecting organisms (2). Sepsis and SIRS activate immunity through similar (or identical) pattern recognition receptors but distinguishing between them clinically is critical, perhaps most obviously because empiric use of antibiotics encourages the emergence of resistant bacteria.

The sharing of cellular pathways by which DAMPs and PAMPs act can cause the downstream immune responses to sepsis and SIRS to be indistinguishable. Thus, clinical responses to infective and non-infective challenge may also be similar (3). Current clinical practice relies on laboratory tests and clinical judgment to distinguish between sterile SIRS and sepsis due to pathogens. Cultures diagnostic of infection typically take days to grow out, limiting their value. Moreover, in many cases bacterial isolates only reflect colonization. Last, infective and non-infective inflammation can co-exist. Clinical examples are frequent, but this is commonly seen after trauma, major surgery or tissue injury from processes like gout.

Mediators of inflammation have been advanced as biomarkers to discriminate between sepsis and sterile SIRS. But since the pathways activated by sepsis and SIRS are shared the mediators produced are often identical. This limits diagnostic value (4).

We have previously used PCR-based assays specific for mitochondrial DNA (mtDNA) to demonstrate the presence and severity of sterile tissue injury (3, 5) and broad-spectrum bacterial DNA (bDNA) assays can be used to detect the presence of pathogens in sepsis (6). We hypothesized that simultaneous assays of mtDNA and bDNA might help categorize the pathogenesis of SIRS in a way that would allow rapid, informed clinical decisions.

To test these hypotheses in a clinically relevant way, we assayed bDNA and mtDNA in stored plasma samples from non-human primates (*Papio* baboons) that had been subjected either to bacterial sepsis or noninfectious toxin challenge. Samples were archived from prior and ongoing collaborative studies in sepsis, inflammation, SIRS responses and therapeutic approaches (7-9). Progressive lethal bacterial sepsis was induced by infusion of *Bacillus anthracis* deltaSterne ($pXO1^{-}pXO2^{-}$), a non-toxigenic model of late stage anthrax sepsis. The contributions of sepsis and secondary inflammation to organ dysfunction and outcome were then further differentiated by treating baboons with activated protein C (aPC; drotrecogin alfa, activated) prior to anthrax infusion. The effects of aPC are incompletely understood and likely multiple, but it has been shown to provide micro-circulatory anti-coagulation and protection against endothelial injury (10). aPC also acts directly on immune cells to suppress innate immunity (11, 12). To contrast lethal anthrax sepsis with another form of non-lethal sepsis we used sera from an *E.coli* infusion model of sepsis where SIRS spontaneously resolves after termination of the infusion. Last, to contrast sepsis to truly sterile SIRS, tissue injury was induced by intravenous injection of Shiga-like toxin 1 (Stx1). This ribosomal toxin (13) is a primary virulence factor of non-invasive enterohemorrhagic

E.coli that causes hemolytic uremic syndrome and acute renal failure (8, 14). In these patients, antibiotic treatment can cause harm by activating phage sequences that up-regulate toxin production (15).

Thus our group of models generally allow us to quantify DAMPs and PAMPs in 1) sterile tissue injury, 2) self-limited bacteremia, 3) bacteremia that progresses to death due to SIRS after sterilization and 4) identical bacteremia where SIRS and death are prevented by aPC rescue. Each model has advantages and limitations and it is the combination of methods that yields more important, generalizable results.

In addition to evaluating mtDNA and bDNA as biomarkers to distinguish sepsis and sterile SIRS, we hypothesized that bDNA levels might improve precision in defining the presence and course of bacteremia, and that mtDNA levels might quantify tissue injury caused by either septic or sterile insults. Last, we speculated that studying correlations between these biomarkers and clinical indicators of organ dysfunction might help define the contribution of bacterial PAMPs and endogenous DAMPs to clinical organ dysfunction in sepsis and sterile SIRS.

MATERIALS AND METHODS

DNA Isolation

Thawed plasma used for PCR analyses was spun twice at 3000xg to remove residual cells, platelets, micro-particles and debris. DNA was prepared from 100µL plasma using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, except that 80µL volume was used to elute DNA from the spin columns.

Real-time PCR Protocols

Cytochrome B primers for mtDNA were chosen for study because unique among mitochondrial molecules, cytochrome B is essentially absent from bacteria on BLAST study (3). Similarly, PCRs targeting 16S bacterial ribosomal RNA have long been used as broad spectrum probes for bacteria. We noted however, that 12s mitochondrial RNA bears many similarities to bacterial 16S-RNA, creating the possibility of false positive assays. We therefore chose16S-bDNA targets that were universal in Gram-positive, Gram-negative and anaerobic bacteria but were evolutionarily distant from mtDNA ribosomal sequences. It should be noted that fungi have mitochondria and these methods are therefore inappropriate for evaluating fungal sepsis.

The same amount of DNA (5μL) was used for each real-time PCR reaction using SYBR Green Master Mix (Applied Biosystems) by Mastercycler EP Realplex (Eppendorf), StepOne Plus (Applied Biosystems), or Mx3000P (Agilent Technologies). Cytochrome B qPCR was accompanied with a standard curve of mtDNA prepared from baboon liver mitochondria isolated using standard methods. Primers (Invitrogen) for targeted species-specific cytochrome B DNA did not cross-react with bacterial 16S DNA (Table 3).

To reduce variability due to reagent and equipment differences, DNA standard curves were included on each plate using either commercial *E. coli* DNA (Invitrogen) or mtDNA purified from freshly prepared mitochondria using the Wako mtDNA Extractor CT kit. qPCR assays demonstrated high sensitivity and specificity for human mtDNA and bDNA from Gram-positive (*Staphylococcus aureus*), Gram-negative (*E. coli)* and anaerobic (*Bacteroides fragilis*) organisms (**Supplemental Figure 1**). In all cases there was essentially no cross reactivity although the primers are sensitive to fg/mL levels.

Supplemental Figure 1 and Table 1 provide supporting data about the qPCR methods and physiological interpretations.

Animal Ethical Considerations

Animal studies were performed under the oversight of the Institutional Animal Care and Use Committees and Institutional Biosafety Committees of the Boston University School of Medicine, University of Oklahoma Health Sciences Center, and Beth Israel Deaconess Medical Center where appropriate. All studies were performed in compliance with applicable National Institutes of Health and ARRIVE guidelines.

Animal Procedures

All nonhuman primate experiments were performed using methods previously described (8, 9). Methods are summarized here to clarify the different experimental designs and reagents used for challenge or therapeutic purposes. Physiological data and assays of bDNA and mtDNA were performed on archived samples from prior and ongoing studies in sepsis, inflammation and SIRS responses that are part of larger on-going collaborative studies (7-9). *Papio c. cynocephalus* or *Papio c. anubis* (4~6kg) were purchased from the Baboon Research Resource at the University of Oklahoma Health Sciences Center and were free of tuberculosis with leukocyte counts <15,000 mm^3. Briefly, the baboons were fasted for 24 hours before the study, with free access to water. They were immobilized the morning of the experiment with ketamine (14mg/kg, i.m.) and sodium pentobarbital administered through a percutaneous catheter in the cephalic vein of the forearm to maintain a light level of surgical anesthesia (2 mg/kg, about every 20–40 min). Baboons were orally intubated and positioned on their left side on a heat pad and catheterized as described below for each challenge. Except

for samples for bacteria colony counts, blood samples were collected into 1/100 volume of 5,000 U/ml penicillin and 500 µg/ml streptomycin. Citrated plasma was stored at -80^{o}C before analysis.

Animals were monitored continuously for disease development as described previously (8, 9, 16) and euthanized according to approved criteria when indicated. Vital signs including temperature (T), respiratory rate (RR) and heart rate (HR) were monitored. Complete blood counts (CBC) were determined with a Horiba ABX Micros 60 Hematology Analyzer (Horiba, Irvine, CA). Fibrinogen levels were determined by reference to a standard curve using the KC4 coagulation analyzer. For the standard curve, bovine thrombin is added to a reference plasma of known fibrinogen content and the clotting time is inversely proportional to the fibrinogen content. Plasma chemistry analyses were performed by IDEXX Laboratories (Westbrook, ME). Normal baboon values (mean ± S.D.; from 37 healthy animals) are as follows: WBC 8.1 ± 2.5/ µl; RBC, 5.1 ± 0.4 x 10^{6}/µl; platelets, 276.2 ± 52.5 x 10^{4}/µl; BUN, 14.2 ± 3.0 mg/dL; creatinine, 0.5 ± 0.1 mg/dL; AST 50.8 ± 19.3 U/L; LDH 310.4 ± 72.1 U/L; total protein 5.9 ± 0.5 g/dL; albumin 3.8 ± 0.3 g/dL; APTT, 40.4 ± 5.8 seconds; hematocrit 38.6 ± 2.8 %; hemoglobin 10.9 ± 1.1 g/dL. Animals surviving to Day 7 post-challenge were considered to be long-term survivors. All 7-day survivors were visibly recovered and had no clinical appearance of illness.

Toxin Challenge

Purified recombinant Stx1 was provided by Dr. Vernon Tesh (Department of Microbial and Molecular Pathogenesis, Texas A&M University System Health Science Center, College Station, TX) and was prepared from cell lysates obtained from *E. coli*

DH5α harboring plasmid pCKS112, which contains the *stx1* operon under control of a thermoinducible promoter (9, 17). Stx1 was purified from cell lysates by sequential ion exchange and chromatofocusing chromatography. Purity of toxins was assessed by SDS-PAGE with silver staining and by Western blot analysis. Prepared toxins contained <0.1 ng endotoxin per ml determined by *Limulus* amoebocyte lysate assay (Associates of Cape Cod, Inc., East Falmouth, MA).

An indwelling catheter was placed in the forearm cephalic vein for bolus infusion of toxin (1-2mLs). A second catheter was inserted into the femoral vein by venous cutdown and secured subcutaneously by an internal injection cap (Braun) where it remained for the rest of the study period and was used for blood draws, infusion of saline to replace insensible loss, central venous pressure monitoring, and anesthesia. All animals received Baytril (enrofloxacin; 10mg/kg i.m.) prior to cutdown and catheter placement on Day 0. Baboons then receive either prophylactic Levaquin (levofloxacin; 3.5mg/kg i.v. bolus) or Baytril (10mg/kg i.m.) each day over the experimental period. Animals were weighed daily and toxin-induced hypovolemia was controlled with isotonic saline using criteria developed in previous studies (18).

Bacteria Challenge and aPC Infusion

The cephalic vein catheter was used to infuse *E.coli, B. anthracis* bacteria or sterile saline to replace insensible loss and to monitor central venous pressure and anesthesia level. An additional percutaneous catheter was inserted in the saphenous vein in one hind limb and the catheter advanced to the inferior vena cava; this catheter was used for sampling blood. *E.coli* B7 O86a:K61 (19) ATCC 33985) or *B.anthracis* deltaSterne strain (pXO1-pXO2-) were grown in broth overnight with mixing, collected

by filtration, washed and re-suspended in sterile saline before infusion. Live bacteria were quantitated using the BacTiter-Glo^{TM} Microbial Cell Viability Assay (Promega, Madison, WI) before infusion to confirm challenge dose (9). Blood was drawn at T0, followed immediately by *E.coli* (5x10^9 CFU/kg; n=3) or *B.anthracis* deltaSterne infusion (0.7~3 x 10^9CFU/kg) over 2 hours, typically at 0.2ml/min. After infusion, *E.coli* challenged animals did not receive additional treatment. Animals to receive *B.anthracis* deltaSterne were randomized to receive either bacteria alone (n=4) or pre-treatment with activated protein C (aPC, drotrecogin alfa, activated; Eli Lilly and Co., Indianapolis, IN) followed by bacteria challenge (n=4). Animals treated with aPC received a bolus injection of 3mg/kg at T (-10min). A two-hour bacterial infusion then was initiated at T0hrs with concomitant infusion of aPC at 64 ug/kg/min for 6 hours. *B.anthracis* +/- aPC animals received levofloxacin (7mg/kg over 1 hour) four hours after the start of the bacterial infusion and daily thereafter. This antibiotic regimen was included to more closely mimic a clinical setting.

Bacteremia was confirmed by traditional plating methods using blood obtained at T2hrs just after finishing the infusion and at T4hrs, just before the antibiotics were given. Colony counts varied according to the loading dose. For a 1x10^9 CFU/kg challenge, colony counts were near 1x10^4 CFU/ml at T2hrs and 100 CFU/ml at T4hrs. Colony counts on blood sampled between days 2 to 7 were consistently negative on all animals.

Data Analysis

***Statistics*.** Data were analyzed for differences between baboon groups using Student's T-test, assuming equal variance. To identify the relationships of plasma mtDNA and

bDNA to organ function in the anthrax baboon model, Pearson Correlation coefficients were calculated using SPSS between mtDNA and bDNA levels and the clinical measurements. **Supplemental Table 1** shows the how the clinical variables correlated with mtDNA and bDNA levels. Only those relationships found to have R^2 values with a $P<0.01$ on initial analysis were subjected to further analysis. Post-challenge survival differences due to aPC treatment were calculated with the log-rank (Mantel-Cox) test.

RESULTS

Bacteremia and bacterial DNA in lethal anthrax sepsis

Infusion of attenuated *B.anthracis* in this nonhuman primate model results in high-grade bacteremia similar to that seen after germination of inhaled anthrax spores (20, 21) and produces septic responses typical of those seen in clinical experience (21) and experimental modeling (9, 22, 23). Anthrax exotoxins are known virulence factors independent of bacteremia (24). To study the specific contribution of sepsis to organ failure and death, baboons were challenged with an anthrax strain (deltaSterne) altered to remove exotoxins (25). The contribution of endogenous inflammation to the sepsis syndrome was assessed using aPC, which attenuates progression of sepsis to organ failure (severe sepsis) and death in baboons without an effect on bacteremia *per se*.

More than 10^4 CFU/ml of bacteria were found in plasma at the end of the infusion **(Fig 1)**. Antibiotics were administered 2 hours after the end of the bacterial challenge and daily thereafter. Bacterial culture counts fell rapidly after antibiotic administration and were negative by 24 hours in all animals. Pre-treatment with aPC had no effect on quantitative blood cultures.

Bacteremia was also readily identified using qPCR for 16S bacterial DNA **(Fig 2A)** isolated from plasma and amplified using primers that have no cross reactivity with mitochondrial ribosomal DNA (see **Supplement Fig 1**). At 10 hours post-challenge, bDNA levels were elevated at 69.9 ± 4.2ng/ml. As with the quantitative blood cultures, pre-treatment with aPC (dotted line) had no significant effect on bDNA load. bDNA titers fell rapidly after antibiotic treatment, but detection of bDNA was still more sensitive than blood cultures. bDNA was still easily detectable at ≥10 ng/mL in plasma at 24 hours ($P<0.01$ vs basal 4.2 ± 1.0ng/ml). bDNA was undetectable in plasma by 48 hours post-challenge.

Mitochondrial DNA in lethal sepsis

mtDNA levels rose markedly in parallel with bacteremia in all animals infused with *B.anthracis* deltaSterne, showing that anthrax sepsis caused tissue injury directly and immediately. mtDNA levels during anthrax bacteremia peaked by 10 hours post-challenge at approximately 200 ng/mL either with or without aPC pre-treatment (**Fig 2B**). In animals not treated with aPC, mtDNA levels remained persistently elevated (solid line) at 48 hours and beyond, well after bacteria and bDNA disappeared from the blood. All these untreated animals progressed to irreversible multiple organ failure and were non-survivors (**Fig2C)**. Baboons pre-treated with aPC (dotted line) were exposed to the same initial bacteria challenge. In contrast, their plasma mtDNA levels decayed rapidly after their bacteremia resolved. Thus tissue injury did not persist after resolution of the bacteremia and all of these animals survived. Since the groups cleared

bacteremia similarly (**Figs. 1,2A)**, this suggests that in this model, aPC prevented persistent endogenous inflammatory tissue injury after bacteremia was cleared.

Bacterial and mitochondrial DNA in sterile tissue injury

Shiga-like toxin 1 (Stx1) is a ribosome-inactivating toxin that causes progressive cellular injury and organ injury, particularly in the kidneys. Stx1 challenge has been characterized extensively in this baboon model and leads to death from hemolytic uremic syndrome and acute renal failure (8, 16) . Stx1 itself is sterile,so its administration will cause toxic rather than infective tissue injury. After high dose Stx1 challenge, plasma mtDNA levels progressively increased as the animals' conditions deteriorated (**Fig.3**). At this dose (100ng/kg), renal injury is accompanied by increasing plasma BUN and creatinine, and decreasing urinary output and loss of glomerular function, leading to acute renal failure and euthanasia by day 3 post-challenge (8). Circulating bDNA levels were insignificant (**Fig.3**) confirming that cellular injury and death after Stx1 was independent of bacteria due to exogenous infection or indirect endogenous sources like gut translocation of bacteria (26).

Bacterial and mitochondrial DNA in sub-lethal *E.coli* infection

To further evaluate the spectrum of tissue injury in infection, we quantified circulating bDNA and mtDNA in a well-characterized model of sub-lethal *E. coli* bacteremia. Baboons respond to this challenge with fever, tachypnea and transient leukopenia. The SIRS response is self-limited however, as judged by the transient cyokine and complement activation and the mild increases in liver function tests that are seen (27-29). As with lethal anthrax sepsis, bDNA became markedly elevated during the bacterial infusion phase **(Fig.4)** and its concentration subsequently decayed rapidly as circulating

bacteria were cleared by the animals. Also as with lethal sepsis, plasma mtDNA concentrations paralleled bDNA levels during the bacteremia itself. Critically though, the mtDNA injury signal disappeared immediately after the cessation of infusion, returning to baseline even before bDNA was totally cleared. This contrasts strongly with events seen in the lethal sepsis model: there, tissue injury signals persisted after bacterial clearance; right up to the time animals were euthanized **(Fig 2B)**. These observations suggest that a self-perpetuating SIRS response due to release of cellular DAMPs including mtDNA (3) is initiated by lethal, but not by non-lethal bacteremia. These findings also suggests mtDNA may be an appropriate biomarker in such cases that reports the severity and outcome of infectious SIRS.

Multiple Organ Failure in SIRS versus Sepsis

The above data show that bDNA is a PAMP and that its levels correlate with bacteremia *per se* whereas mtDNA is a DAMP that reports tissue injury from SIRS. We investigated this in more detail using physiologic data from the *B.anthracis* deltaSterne model that was lethal unless the animals were protected with aPC pre-treatment.

Cardiorespiratory dysfunction

Heart rate and respiratory rate were monitored as clinical markers for the effects of anthrax sepsis on the heart and lungs. Both markers rose with the onset of bacteremia. Regression analysis shows changes in respiratory rates over time are closely related to mtDNA concentration **(Fig 5b),** and not to bDNA concentration **(Fig 5a)**. Respiratory rates did not vary with bDNA and simply increased over time in this lethal sepsis model. With aPC administration, respiration rates remained normal irrespective of DAMP or PAMP concentrations, and in all animals aPC prevented

tachypnea. Thus, aPC altered the respiratory response to inflammatory stimuli, not simply to bacteremia. This suggests that it is tissue injury rather than the presence of bacteria that drives tachypnea in this severe sepsis model. It also raises the possibility that the persistent circulation of DAMPs drives respiratory responses to sepsis after bacterial PAMPs are cleared.

Hepatic and renal dysfunction

Liver and kidney function **(Fig 6)** were markedly spared in anthrax challenged animals pretreated with aPC, consistent with declining mtDNA concentrations (**Fig 2B**). aPC blunted increases in plasma transaminases (AST, ALT) reflective of hepatocellular injury **(Fig 6 A,B)** and increases in blood urea nitrogen (BUN) and creatinine reflective of kidney injury **(Fig 6 C, D)**. Without treatment, all these markers of septic solid organ injury showed dramatic increases after anthrax infusion beginning about 6 hours post-challenge and continuing through 24 hours. Lesser effects on organ function occurred after aPC despite the cohorts clearing bacteria and bDNA identically. Decreased solid organ dysfunction after aPC treatment paralleled diminished circulating mtDNA. This suggests endogenous responses to sepsis cause can cause a substantial amount of the cellular injury in this model of anthrax sepsis which has previously been attributed to the presence of the bacteria.

Hematologic dysfunction

Plasma fibrinogen (**Fig. 7A**) and platelet levels (9) fall early in high-grade sepsis complicated by disseminated intravascular coagulation. Fibrinogen levels usually then rebound as a reflection of the hepatic acute phase response. Sepsis induced by *B.anthracis* deltaSterne led to a rapid and precipitous fall in fibrinogen during the period

of bacteremia itself, followed by slow recovery. aPC is a strong anti-coagulant and it significantly mitigated decreases in fibrinogen ($p<0.0001$ at T4,6,10 hrs). mtDNA concentration did not correlate with plasma fibrinogen levels (**Fig 7C**) or platelet levels (data not shown) regardless of treatment. Rather, fibrinogen levels were linked to the presence of bDNA (**Fig 7B**). Thus, the presence of bacterial PAMPs may be specific contributory factors for the induction of disseminated intravascular coagulation in this severe sepsis model.

DISCUSSION

Sepsis can kill the host through residual SIRS long after pathogens have been cleared. PAMPs and DAMPs are the molecular instigators of SIRS responses to pathogens and tissue injury, but similarities in the inflammatory pathways they activate mean that continued infection, SIRS due to sterile injury or SIRS due to earlier sepsis can look very similar. We studied this problem by comparing circulating DAMPs and PAMPs in non-human primate models of 1) antibiotic treated but nonetheless lethal anthrax sepsis, 2) identical lethal anthrax sepsis with aPC attenuation of endogenous responses, 3) sub-lethal *E. coli* sepsis and 4) lethal sterile tissue injury due to Stx1. We used bacterial 16S-rDNA as our biomarker for bacterial sepsis. Mitochondrial cytochrome B DNA was our biomarker for cellular injury, but it is also a DAMP that initiates inflammation independent of bacteremia (3). We show here using these biomarkers that treated lethal sepsis can be resolved into an initial period of tissue injury by the pathogen followed a period of sterile inflammation. Progressive secondary tissue injury, sustained release of DAMPs and death were averted by aPC. Suppression of

DAMP release also correlated with diminished organ injury and survival from otherwise lethal bacteremia. Similarly, progression of SIRS and death after bacterial clearance were not seen in the self-limited *E.coli* infection where DAMP release stopped spontaneously after bacteremia cleared.

The data show pathogens cause SIRS and organ dysfunction directly, but that when severe enough, bacteremia left ongoing residual tissue injury in its wake that was reflected by mtDNA. There was increasing mtDNA but essentially undetectable bDNA levels in the animals challenged with Stx1, supporting the discriminatory value of the two biomarkers in these settings. Control studies **(Suppl Fig 1)** showed detection thresholds for bDNA were about 100 fg/mL without cross-recognition of mtDNA. This level of sensitivity and specificity will be needed if we are to use these PCR assays for clinical discrimination between sepsis and SIRS.

The usual cause of morbidity and mortality after sepsis and SIRS is multiple organ failure. In this nonhuman primate model of high-grade sepsis, clinical diagnostic blood tests reflect organ injury as assessed by direct pathologic examination (9). We observed here that aPC pre-treatment had no effect on bacterial PAMP load (Figs 1,2A) but did decrease DAMP load and lessened evidence of organ failure. Pre-treatment with aPC also helped us resolve the contributions of bacteremia *per se* and endogenous SIRS to the organ injury initiated by bacteremia.

We investigated these relationships further by mathematically analyzing the correlation between mtDNA and bDNA concentrations and clinical markers of organ injury. We did this over time both with and without aPC rescue. The mechanisms of aPC protection are incompletely understood, but aPC has both anti-coagulant and direct

cytoprotective activities (30, 31). Either of these actions could improve tissue perfusion or reduce inflammation and thus contribute to the decreased cellular injury seen. After preliminary assessment of Pearson correlation coefficients (see Suppl. Table 1), significant correlations between biomarker concentration and organ injury ($p<0.01$) were subjected to further analysis**.** Respiratory rate is a simple assessment of early acute lung injury. It becomes a complex variable late in sepsis as animals become acidotic and develop ventilator and renal failure, but intervention with aPC altered the relationship between infection and respiration rate in a fundamentally unexpected way. Respiratory rate (RR) never correlated with bDNA (or bacterial counts) (**Fig 5**). In aPC-untreated animals, RR simply increased until terminal decompensation. With aPC treatment, RR was simply unchanged even though bDNA rose and fell during the acute bacteremia phase. In contrast, respiration rates varied directly and significantly with mtDNA levels in untreated animals. With aPC pre-treatment, this link was broken and respiration rates failed to vary with mtDNA. These findings suggest DAMPs from septic injury, rather than the bacterial PAMPs that drive DAMP release, may cause tachypnea in this model. An opposite pattern was seen examining the hematologic manifestations of severe sepsis and SIRS. At no time did plasma fibrinogen levels bear any relationship to mtDNA **(Fig 7C)**, whereas fibrinogen concentration was strongly inversely related to bDNA ($P<0.01$, **Fig 7B**). Thus, bacterial PAMPs appear associated with fibrinogen consumption and disseminated intravascular coagulation, whereas in this model mtDNA DAMPs have minimal association with coagulopathy.

These data generally suggest that when SIRS persists after appropriate anti-microbial therapy it may be due to continuing DAMP release rather than persistent

infection. So whereas sepsis is usually defined as SIRS with reasonable expectation of an invading pathogen being the cause (32), this definition is clearly imprecise and bacterial pathogens are often assumed to be present in clinical SIRS when none are found in cultures. Our results suggest that where infection and tissue injury can be defined with precision by biomarkers it may be more useful to define "infection" as active tissue invasion by pathogens, "SIRS" as the innate immune response to alarmin release from any source and "sepsis" as an infective challenge sufficient to cause SIRS.

Thus antibiotics can kill bacteria but cannot eliminate SIRS and biologic response modifiers (like aPC) may block SIRS by preventing a vicious cycle of cellular injury and DAMP release. Recent studies on extracellular histone DAMPs (33-35) show that their release in response to sepsis can activate TLR2 and TLR4 and that extracellular histone signaling can lead to death. Those mechanistic studies are of particular interest here since histones are pharmacologically targeted by aPC, which cleaves them and reduces their cytotoxicity (35). Yet suppression of inflammation is achieved at the risk of potentiating active or persistent infections (36-38).

So although antibiotics and anti-inflammatory therapies may be complementary, combining them is most likely to improve outcomes if they can be used at a time and in a sequence appropriate to ongoing molecular pathophysiology and anti-inflammatory strategies used incorrectly can blunt needed immune responses to sepsis. Inflammatory organ failure is also exaggerated in baboon sepsis compared with human sepsis, and human sepsis is typically low-grade compared with experimental models. Thus although aPC is effective in mitigating septic organ failure in baboons it has not been found

clinically effective in humans. Thus, the differences between baboon and human sepsis preclude our findings being used to apply aPC directly to human disease.

Experimentally however, we found that using aPC in baboon anthrax was an effective model for discriminating the direct effects of sepsis and secondary inflammation. These experimental conditions also yield insight into the relationships between the severity of sepsis and SIRS as assayed by DNA biomarkers and organ failure. It would be premature to use these biomarkers clinically in humans. Highly structured, timed collections such as those we used here in high-grade animal sepsis models only let us begin to understand the significance of DNA biomarkers. The rapid and marked fluctuations seen early in sepsis explain why “spot measurements” will be difficult to interpret (39) and moreover, long-term persistence of damage signaling rather than its early occurrence may be the precursor of organ failure.

We do propose however, that with further study, biomarkers reflecting the activity of PAMPs and DAMPs may prove important in directing the timing and use of antibiotics and biologic response modifiers in clinical practice. Future studies must test those hypotheses directly in human samples and subjects.

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Figure 1

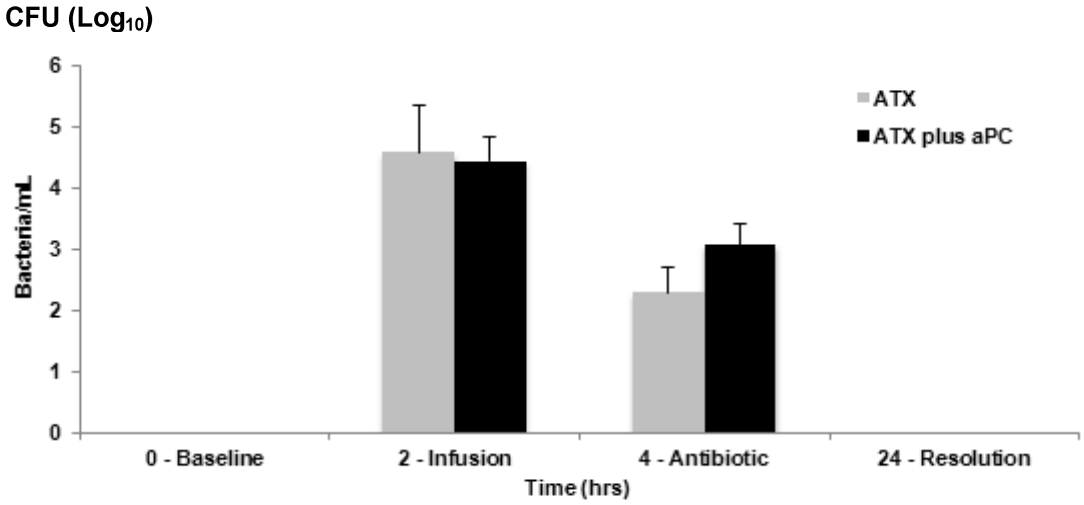

Figure 1. aPC does not alter bacteremia levels. Blood was drawn before (T0hrs) infusion, just at the end of *B.anthracis* deltaSterne infusion (T2hrs) and at T4hrs just before administration of levofloxacin (7mg/kg) and cultured for determination of CFU/ml. Grey bars are without aPC pre-treatment, black bars are with aPC pre-treatment. All animals received antibiotic treatment daily (n=4/group). Colony counts on blood sampled between days 2 to 7 were consistently negative.

Figure 2

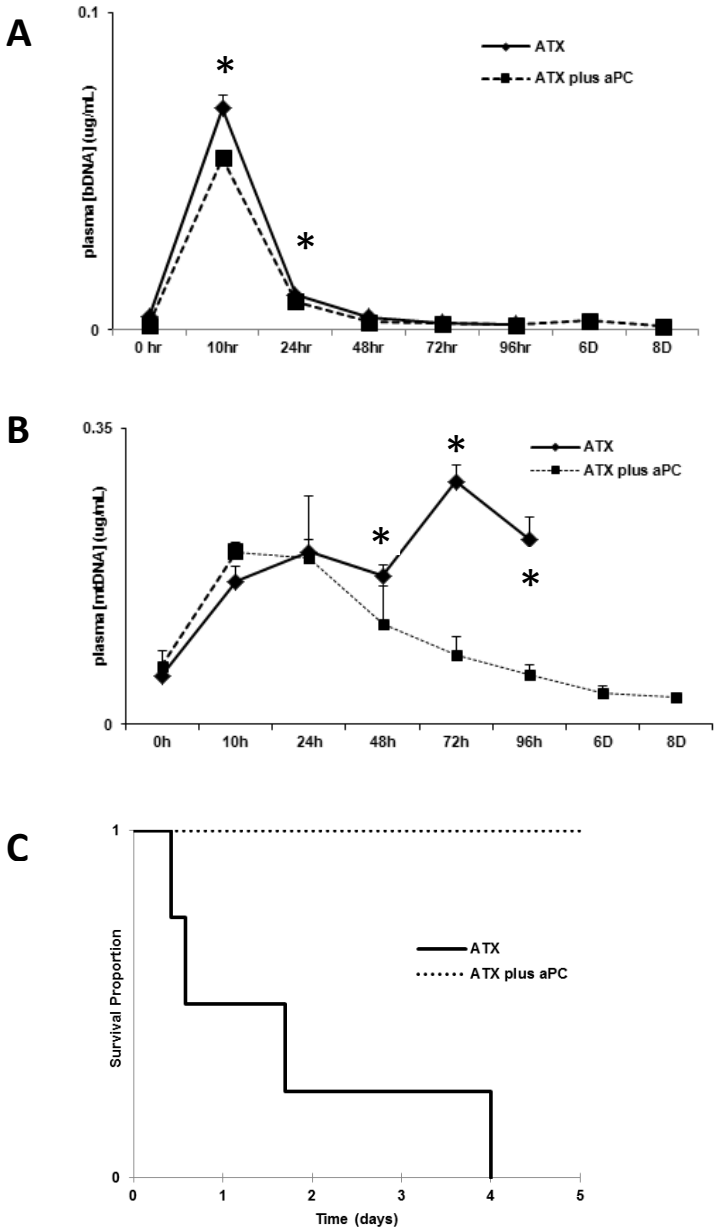

Figure 2. mtDNA changes are consistent with survival outcome. Intravenous infusion of *B.anthracis* results in increased plasma bDNA levels by 10 hours post-challenge (A) which decline but remain detectable up to 48 hours. (B) mtDNA levels peaked by 10 hours, remained elevated without therapeutic treatment, and all these animals required euthanasia by Day 4 post-challenge. aPC treatment prompted reduction in plasma mtDNA levels and resulted in survival of all treated animals (C; Kaplan Meier; n=4/group, **p<0.001**).

Figure 3

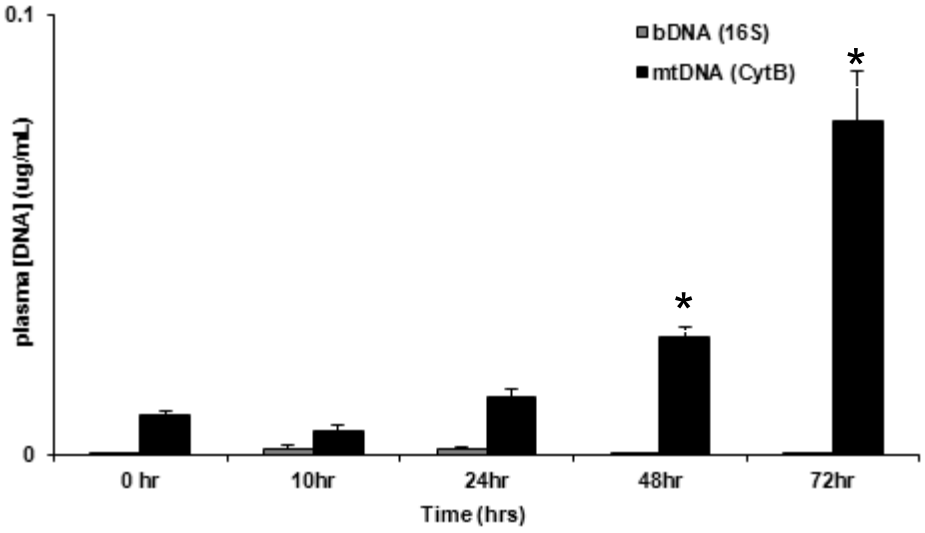

Figure 3. Non-infectious toxin challenge increased only mtDNA. Baboons (n=3) were challenged with purified Stx1 (50ng/kg, i.v.) on Day 0. Prophylactic antibiotic was administered daily. All animals succumbed with acute renal failure and organ damage necessitating euthanasia by 72-74 hrs post-challenge. (* $p<0.05$)

Figure 4

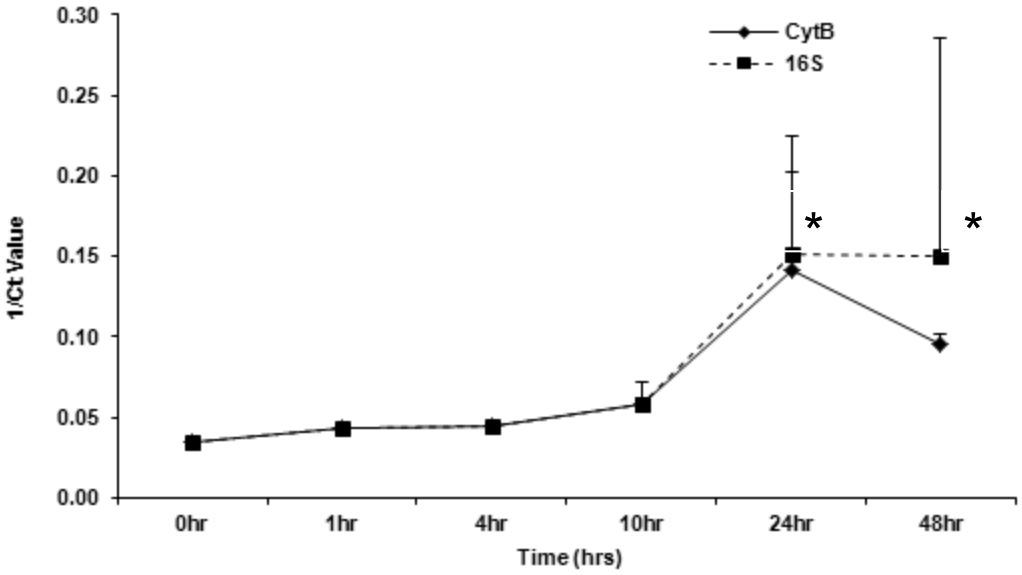

Figure 4. Sublethal septic challenge with *E. coli* B7 O86a:K61. Both bacterial and mitochondrial DNA are detected *after E.coli* infusion of baboons (n=3). The infection is self-limiting with rapid bacterial clearance and reduced mtDNA by 48 hours post-challenge. (* $p<0.05$)

Figure 5

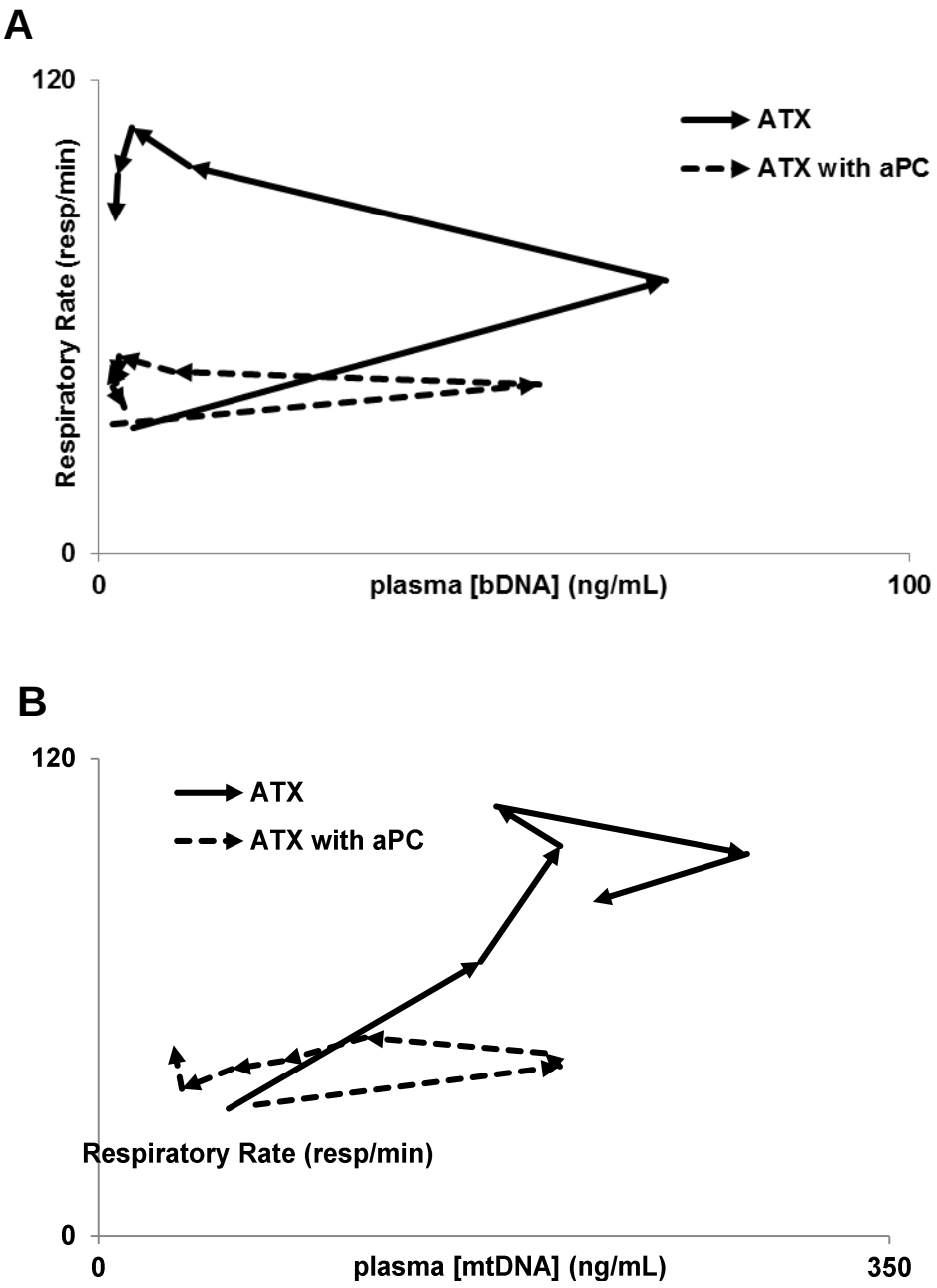

Figure 5. aPC alters the relationship between inflammatory stimuli and respiratory rate over time (→ denotes course of relationship over time here and in Figures 7 & 8). Respiratory rate does not vary with bDNA (above), rather, without aPC, the respiratory rate simply increases over time. In distinction, the respiratory rate does vary with mtDNA concentration without aPC (below, R^2=0.784). In both cases aPC prevents tachypnea.

Figure 6A,B

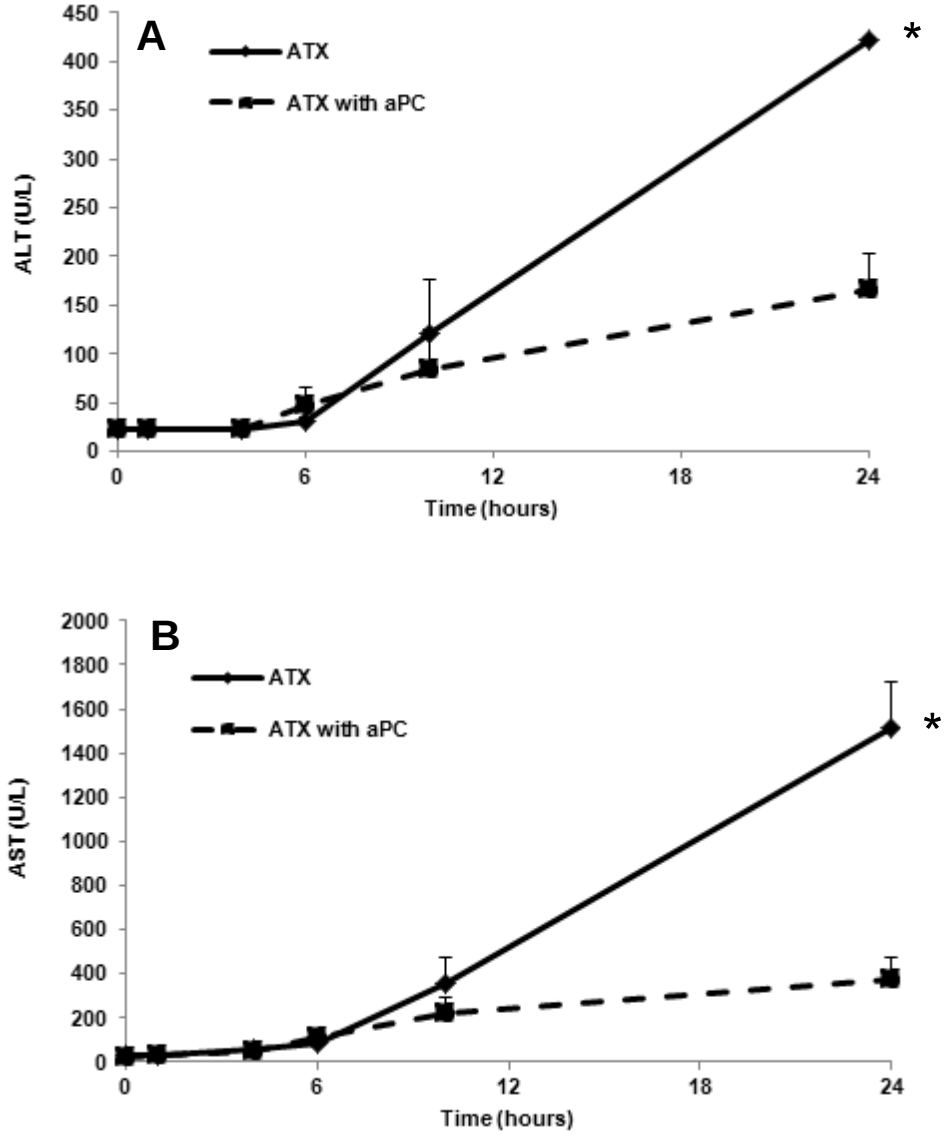

Figure 6. Markers of septic organ injury. Plasma transaminases (A,B; AST, ALT) were measured as markers for hepatocellular injury in sepsis and SIRS over the first 24 hours. Creatinine and blood urea nitrogen (BUN) were measured as markers for kidney injury (C,D). All these markers of septic organ injury showed dramatic increases after anthrax infusion beginning around 6 hours and continuing through 24 hours. Thus, liver and kidney function were progressively compromised in sepsis due to *B. anthracis.*

Figure 6C,D

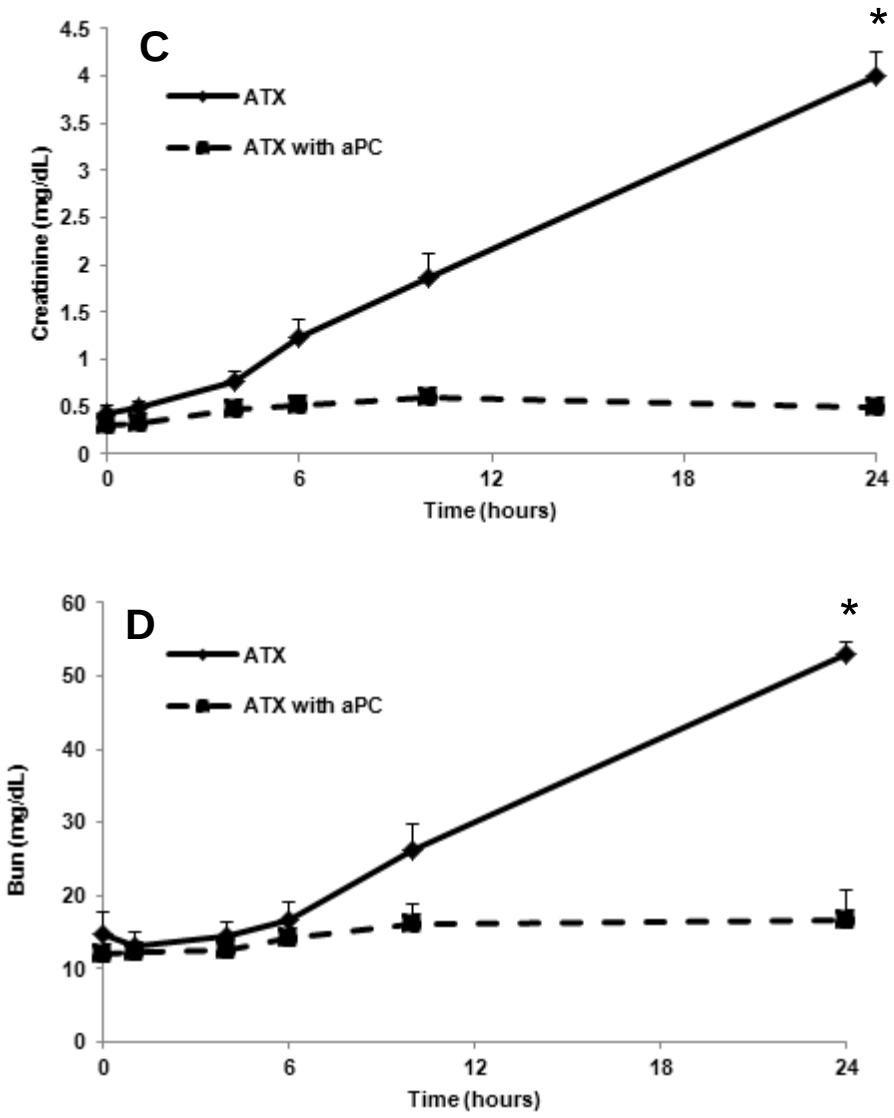

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Figure 7

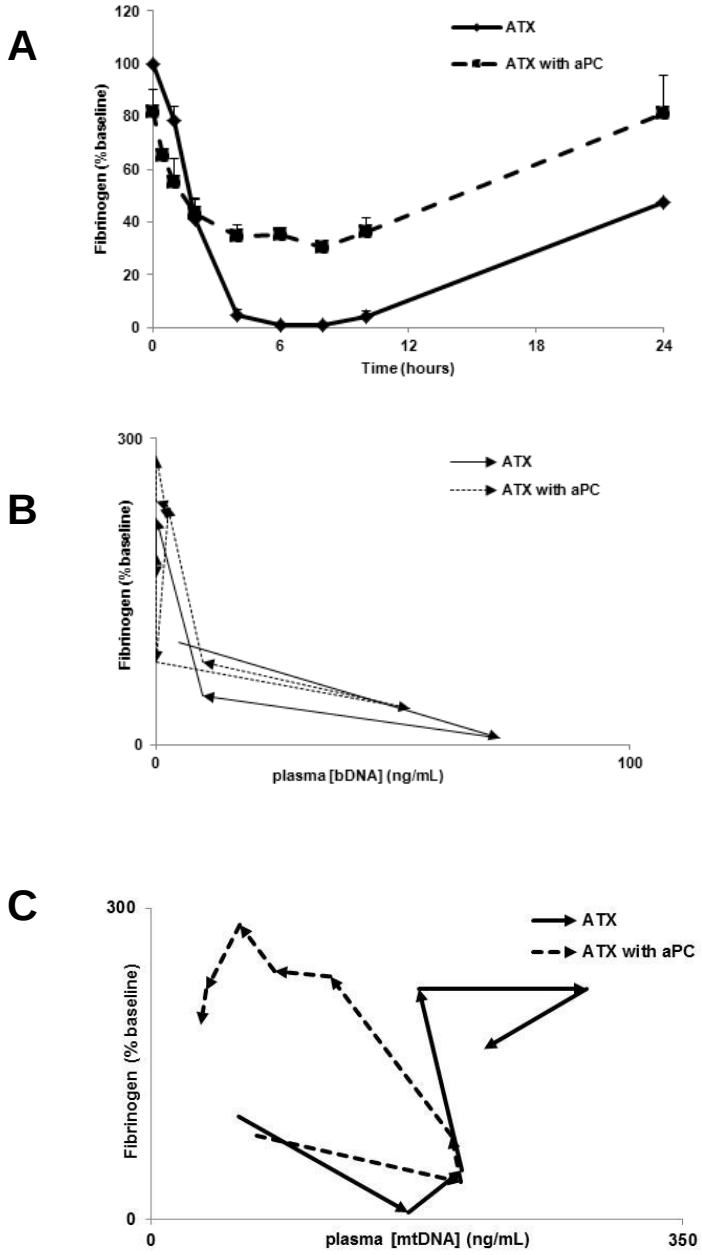
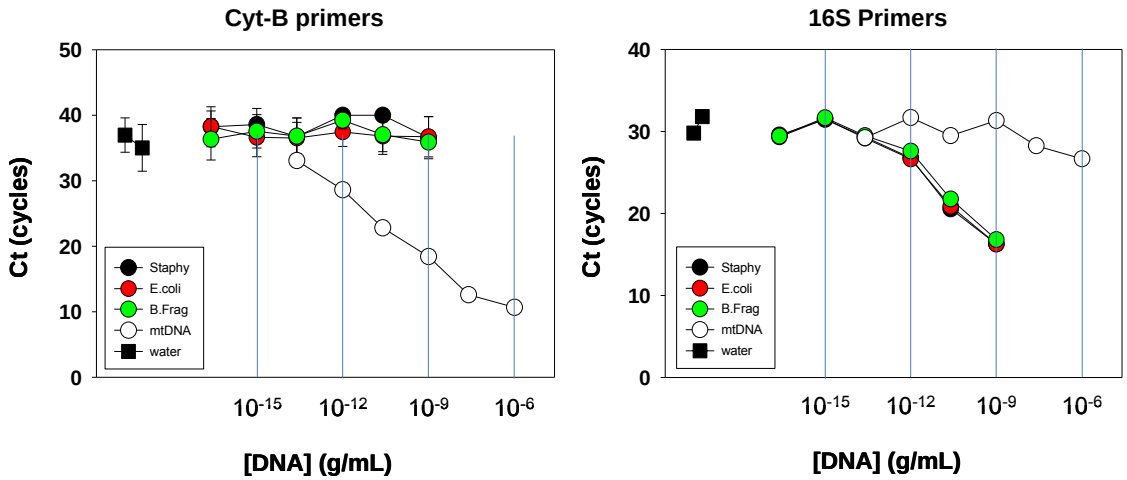


Figure 7. **Disseminated intravascular coagulation is linked to bacteremia.** The consumptive coagulopathy precipitated by anthrax bacteremia depletes fibrinogen rapidly and is mitigated by aPC treatment (A; $p<0.0001$ T4,6,10 hrs) and correlates with bDNA levels (B). Regardless of treatment, mtDNA concentration did not correlate to plasma fibrinogen level (C). Thus, presence of bacteria appears specifically to induce DIC, whereas the inflammatory response to tissue injury as monitored by mtDNA does not cause DIC, even when it is initiated by bacteremia.

Supplemental Figure 1



Supplementary Figure 1. Sensitivity and Specificity of Primers

Controls are shown for cytochrome B and 16S primers, showing sensitivity and specificity for their targets. (A) Cytochrome B primers do not detect any of three distinct kinds of bDNA: Gram-negative (*E. coli*), Gram-positive (*S. aureus*), and anaerobe (*B. fragilis*). mtDNA was able to be distinguished at less than 1 pg/mL. (B) 16S primers do not detect mtDNA, but show equivalent level of detection for all three kinds of bDNA assayed. bDNA was able to be distinguished at less than 1 pg/mL.

Supplemental Table 1

mtDNA	Respiratory Rate	p<0.01
	APTT	p<0.05
bDNA	Heart Rate	p<0.05
	sO_2	p<0.05
	Fibrinogen	p<0.01
	APTT	p<0.01
	WBC	p<0.05
	RBC	p<0.01
	Hematocrit	p<0.01
	Hemoglobin	p<0.01

Supplementary Table 1. Pearson Correlation Coefficients

Pearson Correlation Coefficients were calculated to assess relationships between clinical values and our markers (mtDNA for DAMPs, bDNA for PAMPs). Only those correlations with p<0.01 were subjected to further analysis.

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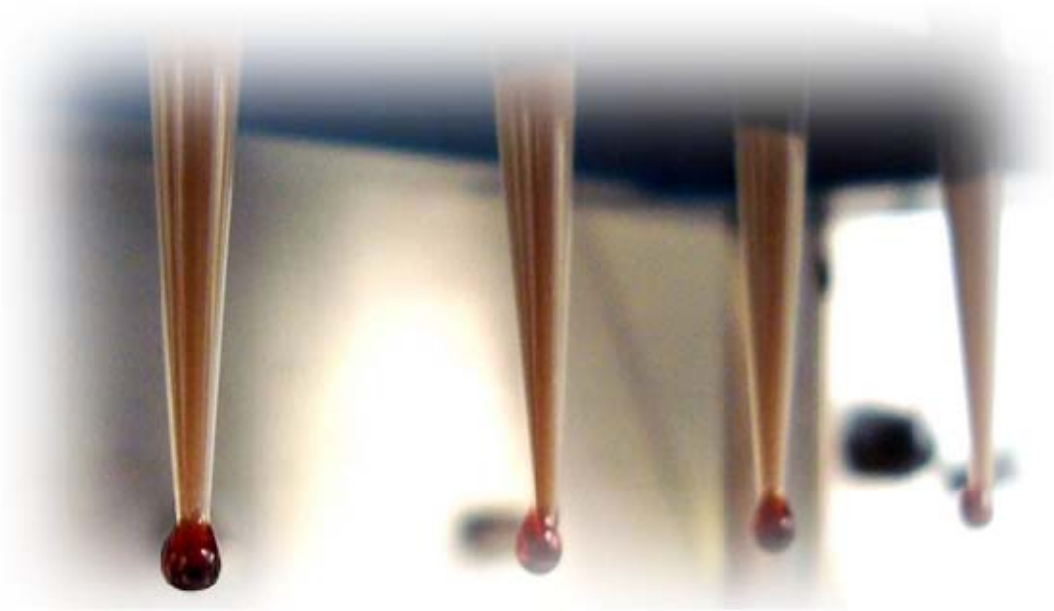
Solid Phase Extraction of mtDNA and E. Coli Cells in Human Blood with a Porous Polymer System



12/17/2010

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BOSTON
UNIVERSITY

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Symbols and Abbreviations

Capital Letters

[…]

1 Proposal (CIMIT)

Background and Significance

1) Mitochondrial Damage patterns are crucial biomarkers for sterile SIRS

Injury and sepsis are two of the leading causes of premature death in the United States [1]. In both syndromes, death and morbidity are linked to the Systemic Inflammatory Response Syndrome (SIRS) [2]. SIRS is the clinical manifestation of globally activated innate immunity [3], and causes organ failure as well as susceptibility to secondary infections. Similarly in sepsis, illness and organ dysfunction persist long after bacteria have been killed by anti-infective agents. **Early differentiation of Sepsis from SIRS will have an enormous impact civilian and military casualty care as well as on the treatment of other medical and surgical illnesses.**

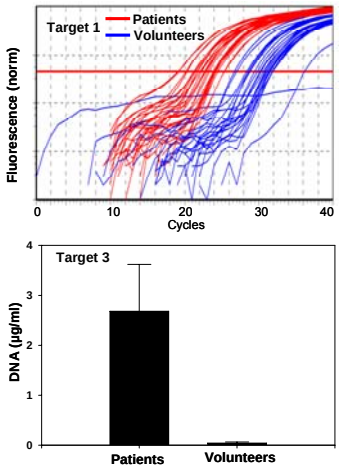

Figure 1 (above) shows qPCRs for the mtDNA gene Cytochrome B in the plasma of severely injured (red trace) patients and matched controls (blue traces). Injury releases ~2^{10} fold (about 1000 times) more mtDNA into the plasma than is seen in controls.

We have recently shown (Zhang et al, ***Nature*** March 4, 2010) that "Damage associated molecular patterns: DAMPs from mitochondrial are released in injury related SIRS **(Fig 1)**. Moreover, the molecular patterns released by injury can act by the same immune pathways as those induced by bacterial infection, thus causing traumatic SIRS to be in many cases, completely indistinguishable from sepsis. The major difference is that in sepsis, innate immune pattern recognition receptors (PRR) are activated by pathogen-associated molecular patterns (PAMPs)[11] expressed on microorganisms where in SIRS, PRR are activated by DAMPs released from cells. In each case though, the same germline-defined PRRs can be activated. Both prokaryotic and mitochondrial peptide synthesis is initiated by *N*-formyl methionine [12]. Thus *N*-formyl peptides of either source activate immunocyte calcium flux via formyl peptide receptors (FPRs) **(Fig 2)** and are potent chemoattractants for human neutrophils (PMN) **(Fig 3)**. These are critical events in the initiation of PMN-mediated organ failure after injury. We also found that formylated peptides from mitochondria can activate important MAP-Kinases and induce release of matrix metalloproteinase-8 **(Fig 4)**, a key mediator of PMN infiltration of the lung as well as secondary activator of chemokine pathways.

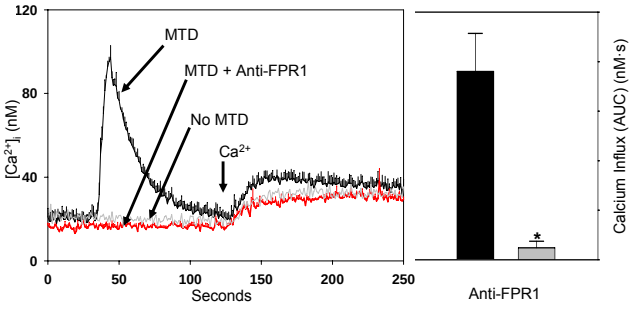

Figure 2 (left): The exposure of Fura-2 loaded PMN to mitochondrial DAMPs (MTD) at t=30s causes calcium release from endosomal stores and store operated calcium entry (SOC) on recalcification of the medium at t=120s (and bar graph). Both are nearly totally inhibited by antibodies to FPR1. Calcium release and re-entry are both critical for chemotaxis, cytokine synthesis and degranulation.

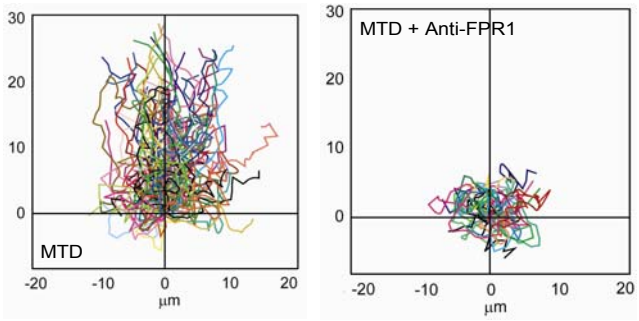

Figure 3 (left): Human PMN respond to mitochondrial DAMPs (MTD) by chemotaxis form their point of origin toward the pipette source (top of figure) in a dark-field video microscope system. The paths of the individual cells are graphed and re-arranged by a computer program. Both distance travelled and directionality are indicators of strength of chemotaxis. We see here that brisk chemotaxis toward the MTD source is blocked by antibodies to FPR1.

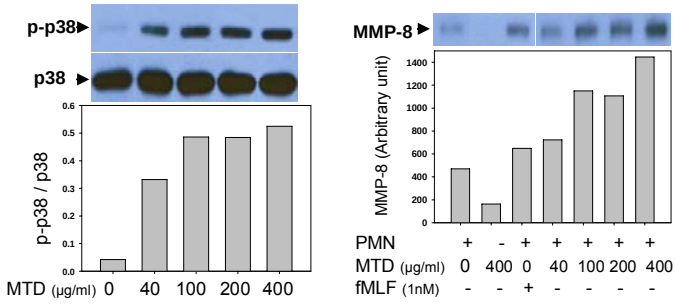

Figure 4 (left): Human PMN activate p38 MAPK (left hand panel) upon exposure to MTD. Also, exposure to MTD induces cells to release MMP-8. MMP-8 release is fully inhibited by antibodies to FPR1 (not shown) or by the FPR1 inhibitor cyclosporin H.

In addition to responding to the formyl peptide components of mitochondria, immune cells like PMN also express Toll-like receptors (TLR) that can respond to inflammatory motifs. Both bacterial DNA (bDNA) and mitochondrial DNA (mtDNA) are high in CpG repeats. We have now shown that both mtDNA and CpG DNA are recognized by PMN and activate p38

MAP kinase (**Fig 5**). p38 MAPK activation is blocked by oligodeoxynucleotides (ODN's) that bind to TLR9 ligands, implicating this key pathway.

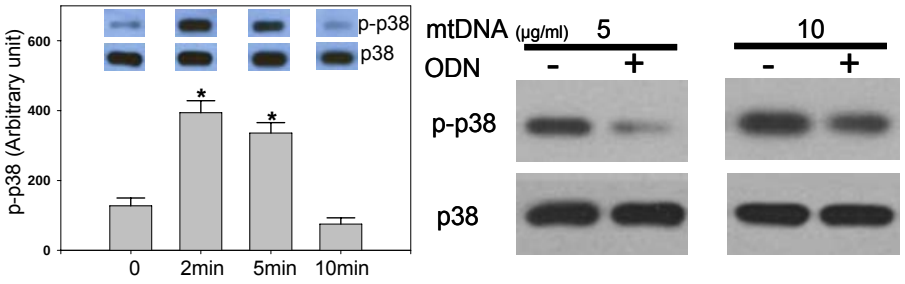

Figure 5 (left): Mitochondrial DNA (mtDNA) rapidly activates PMN p38 MAPK (left). Activation is blocked by ODNs and excess mtDNA overcomes the inhibition by ODNs.

IL-8 secretion by PMN is a crucial event in the development of SIRS and organ failure. We have shown that mtDNA present ***at the concentrations found in trauma plasma*** activates PMN synthesis and release of IL-8 (**Fig. 6**) especially when in synergy with low concentrations of formylated peptides.

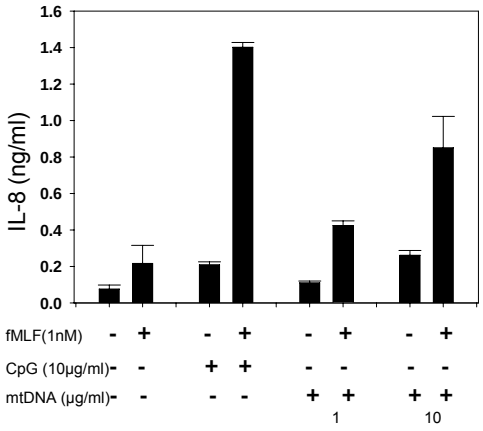

Figure 6 (left): Both mitochondrial DNA (mtDNA) and CpG (bacterial) DNA) cause PMN to secrete IL-8. Activation in each case is increased synergistically by small doses (1nM) of fMLP.

Thus sepsis and SIRS are closely related mechanistically even though they have vastly different 'first causes' and so appropriate early management may be vastly different. The early management of sepsis relies primarily on broad-spectrum antibiotics, which reliably decrease mortality **[Dellinger RP CCM 2004 PMID 15090974]**. Antimicrobial therapy is not helpful in sterile SIRS however, and despite the enormous effort and cost used such therapy leads to the emergence of resistant infections and worse outcomes **[Huttner B Lancet Inf Dis 2010, PMID 20129146 Tenover FC JAMA 1996, PMID 8544270]**. Conversely, anti-inflammatory biologic modification therapies can prevent appropriate responses to invasive organisms. Thus anti-inflammatory therapy often increases mortality in early sepsis **[Fischer CJ, NEJM 1996 PMID, 8637514]** where they might improve outcomes if appropriate patient populations with sterile SIRS could be identified. Diagnostic tests that could discriminate

sepsis and SIRS at an early time point would be therefore expected to improve outcomes from both injury and sepsis, two major causes of morbidity and mortality in injured combatants as well as in general medical / surgical care.

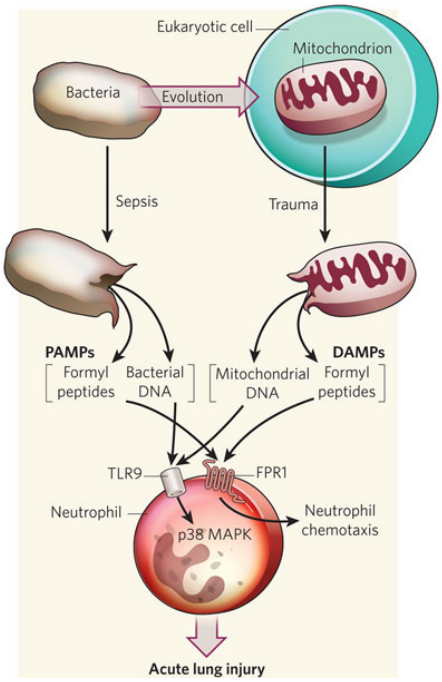

Figure 7 (left): From Calfee and Matthay; Culprits with evolutionary ties. (Editorial) Nature [Volume 464] 4 March 2010. In sepsis and SIRS, PAMPS from bacteria and DAMPs from mitochondria bind to the same receptors and lead to similar clinical responses. Since the DNA ligands involved can be discriminated however, they may offer an opportunity to achieve an early diagnosis that both excludes one syndrome and confirms the other.

Our novel understanding of the early pathogenesis of SIRS now potentially allows us to use PCR-based technologies to discriminate directly between early sepsis and sterile SIRS although both cause similar clinical syndromes by an over-arching similar effect at the level of the initiation of the innate immune response (**Figure 7**).

We now propose to apply our novel understanding of the early danger signals leading to SIRS and Sepsis to the clinical management of these conditions by creating rapid, sensitive and specific tests for mitochondrial and bacterial DNA. These will allow early diagnosis of the responsible agents (ie mtDNA DAMPs or bDNA PAMPs) with a degree of confidence that will allow restriction of antibiotic use in a high percentage of cases. We may later augment PCR testing with testing for peptide products of mitochondrial disruption. **We expect that this testing will lead to vastly improved outcomes in injured patients. These benefits will include less common development of antibiotic resistant organisms, fewer poorly treatable nosocomial infections and considerable subsequent economic savings. Also, as biologic modification therapies become available we will be able to apply them appropriately with less fear of increased mortality due to inappropriate immune suppression in active infections.**

Overall Goals of proposed activity

Development of a POC Test: Based on our novel understanding of differences in the molecular pathogenesis of sterile and infective SIRS we are now developing 'test-tube' methods that can quickly distinguishing sterile from infective SIRS. The Hauser lab has demonstrated that mtDNA for cytochrome B (or other electron transport chain cytochromes) can be easily demonstrated in the plasma and tissue fluids of trauma patients Cyto B primer sequences have no significant homology with DNA found in any bacterial species published on BLAST. Similarly, DNA coding for bacterial 16s-rRNA is not found in patient plasma in the sterile SIRS (**Fig. 8**).

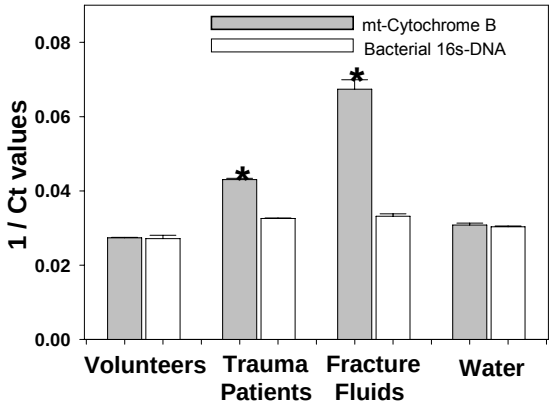

Figure 8: Bacterial nucleic acids cannot be detected by PCR (no difference found from water) whereas the circulation of endogenous mtDAMPs is easily shown by the presence of Cytochrome B in plasma and wound fluids of trauma patients. Zhang et al, 2010 *Nature*, *March 4.*

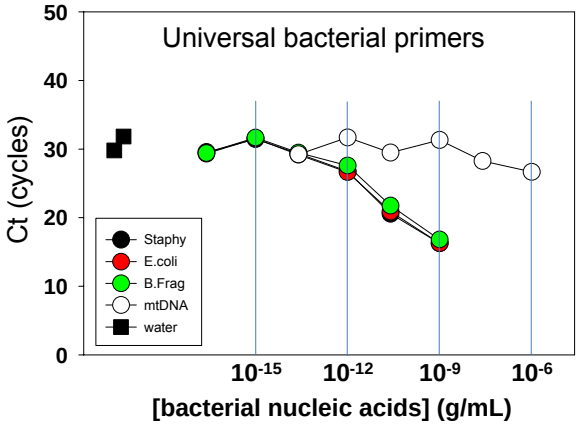

Figure 9: Responses to our bacterial nucleic acid probes are detectable (ie diverge significantly from the 'water-line') for the major pathogens Staph. Aureus (black), E. coli (red) and Bacteroides Fragilis (green circles) at low pg/ml concentrations. Detection is apparently universal and identical across the bacterial phyla. The bacterial probes do not however, detect human genomic nucleic acids or mtDNA (white circles). *Itagaki and Hauser. Unpublished data*.

In additrion, we have now shown that whereas our primer sequences for bacterial 16s-rRNA readily detect authentic DNA from gram-positive, gram-negative and anaerobic bacteria they do not recognize mtDNA **(Figures 8, 9)**. Thus one PCR test can be used to detect the presence of all three major bacterial pathogen groups of interest in the setting of acute trauma without recognizing the mitochondrial genomic equivalent (ie mitochondrial 12s-rRNA).

Thus the Hauser laboratory has defined new bio-markers for SIRS due to tissue injury in the form of mitochondrial DAMPs. These are easily measured and directly responsible for the initiation of inflammation after injury. Of these mtDAMPs, mtDNA has been chosen as an optimal target and "test-tube" PCR methods have been created that can diagnose the presence of sepsis and SIRS using simple comparative testing for mtDNA and bDNA based on primers that are both highly sensitive and highly specific.

We now seek to move these bench top tests closer to the point of care by implementing microfluidic technology to minaturize, speed up, and simplify test protocols for the user. Dr. Klapperich's lab has successfully miniaturized several PCR assays into disposable microfludic devices (including one for influenza A for a prior CIMIT project). These PCR assays incorporate sample preparation steps on chip, in line with the nucleic acid amplification steps.

Nucleic acid extractions take place inside of a plastic microfluidic platform using sample volumes from 100 – 500 microliters. This method has been successfully used to extract PCR amplifiable nucleic acids from mammalian cells (DNA and RNA), influenza virus, gram-positive (*B. subtilis* and *E. faecalis*), and gram-negative bacteria (E. coli) (Mahalanabis et al., *Biomed. Microdev.* 2009). These extractions have been performed using urine, nasopharyngeal washes, stool µLtrafiltrate and whole blood samples. (Gillers, et al., Journal of Microbiological Methods, 2009).

The technology that enables these extractions is based on the formation, using light directed chemistry, of a porous polymer monolithic column inside of the microfluidic device. The high backpressure caused by the small pore size of the column, coupled with a chemical lysis agent, break open cells in the sample, liberating the nucleic acids. The monolith is embedded with second phase particles (silica) to enhance adsorption and concentration of nucleic acids. The captured nucleic acids are washed and eluted in water and can be used directly in downstream amplification assays. The Klapperich Lab has also demonstrated continuous flow PCR (CF-PCR) microfluidic devices containing these porous polymer extraction columns upstream from the amplification area. The on-chip sample preparation combined with the shorter cycling times afforded by working with smaller volumes makes these two systems excellent platforms for point-of-care use.

Specific Methods

To achieve progress therefore towards a POC test discriminating between sterile and infective SIRS, we propose to adapt the test-tube PCR methods developed by the Hauser lab to the on CF-PCR microfluidic platform developed by the Klapperich lab. The technical approach used will initially entail studies using our previously discovered primers:

5'-atgaccccaatacgcaaaat-3' and 5'-cgaagtttcatcatgcggag-3' for Cytochrome B and

5'-cgtcagctcgtgttgtgaaa-3' and 5'-ggcagtctccttgagttcc-3' for bacterial 16s-rRNA.

These will be used in the Klapperich platform which is described below in **Fig 10.**

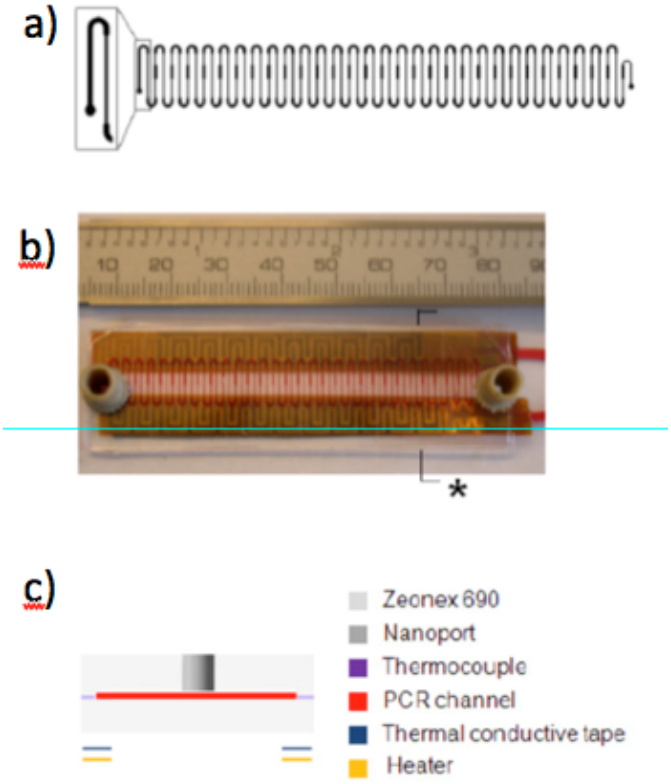

Figure 10:

a) Schematic diagram of a typical continuous flow PCR reaction chamber
b) photograph of a test device
c) cross section of the test device at *, showing the functional layers of the device

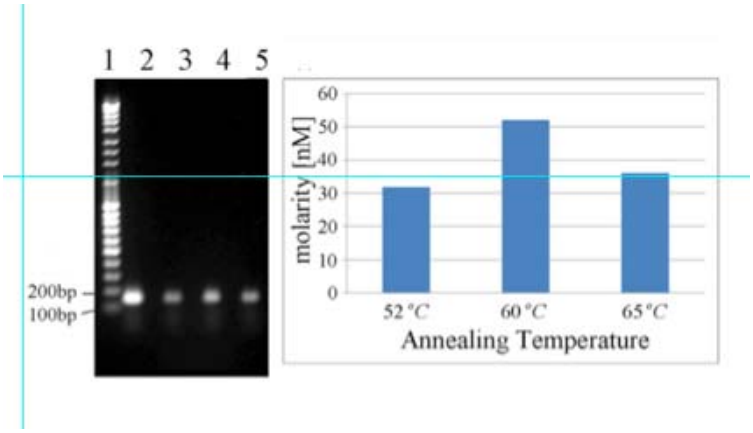

Figure 11: Effects of varying the annealing temperatures on PCR yield in the Klapperich system. The gel shows fluorescence images of on-chip PCR products (Lane 1: DNA ladder, Lane 2: PCR product from commercial thermocycler, Lanes 3-5: on-chip PCR product) at various annealing temperatures, 52˚C, 60˚C, 65˚C, respectively at constant denaturation temperature of **95˚C**. The plots show the endpoint concentrations of the PCR products as measured using an Agilent Bioanalyzer.

Technical approach and Specific Aims

Based on the above novel understandings and technologies, the following studies will be performed and we will:

Specific Aim 1) Develop a chip based assay using primers developed to recognize 16s bDNA and Cyto B in 'authentic' bDNA and mtDNA 'spiked' into platelet-free plasma. Only rarely can a PCR assay designed for tube scale reactions be transferred directly to a microfluidic platform. Necessary adjustments and optimization of reaction conditions, concentrations and blocking agents and amounts will therefore be carried out.

Specific Aim 2) We will then use whole bacteria in plasma and in whole blood with without an external enzymatic lysis step (e.g. lysozyme, proteinase K at elevated temperature). The porous polymer monolithic structure that makes up the chip based solid phase extraction column has an open pore structure with pore sizes on the order of 1-2 microns in diameter. When cells are forced through these pores in the presence of a chemical lysis agent (e.g. chaotropic salt buffer), the mechanical and chemical effects can act to lyse the cells without the need for enzymatic pretreatment. Nonetheless, it may be possible to optimize detection of bDNA in low-grade sepsis by lysing bacteria enzymatically. mtDNA is always detected in platelet-free (ie high-speed spun) plasma since it is expected to be found in all formed cellular elements of the blood.

Specific Aim 3) We will assess the relative sensitivity of the assay for Gram positive (S. Aureus), Gram negative (E. Coli) and anaerobic (B. Fragilis) species. This will be important for structuring subsequent clinical studies.

Specific Aim 4) Perform assays on excess (discarded) blood from specimens used in our hospital microbiology lab for "blood cultures". We will then compare the rates of positive blood cultures and clinical outcomes (by de-identified chart review) of known septic and non-septic (SIRS) patients using the data obtained using our assays.

Impact on clinical care

Availability of an assay differentiating early sepsis from SIRS will have broad impact on clinical care. 1) The vast majority of antibiotic use in the United States is empiric, which is to say that it is given in the absence of a known pathogen or of a proven infection. Our approach will define the need or lack of need for empiric antibiotic treatments in febrile and potentially septic patients. 2) Such decreases in empiric antibiotic use will significantly decrease antibiotic selection pressure on endogenous patient flora. This is expected to decrease or prevent the emergence of resistant flora and resistant infections. 3) Decreased antibiotic use will decrease the frequency of antibiotic related complications like renal toxicity, ototoxicity, allergic reactions (rashes, interstitial nephritis etc) and the development of C. Difficile colitis. 4) Where antibiotic use for sepsis **is** indicated, early demonstration of sepsis by PCR techniques will allow aggressive antibiotic management. 5) Subsequent to the demonstration of sepsis by our methods, more cost-effective early speciation of infective agents by existing techniques or by new techniques that we may build into succeeding versions of our test platform will become feasible. 6) Decreased antibiotic use will result in large decreases in expenditures on antibiotics as well as laboratory costs related to culturing bacteria that are not causing disease and determining their antibiotic sensitivities. 7) The diagnosis of symptomatic SIRS in the absence of invasive infection will allow prompt effective use of anti-inflammatory or biologic response modification therapies in patients who do not have sepsis.

Expected superiority to existing or possible alternatives

As noted above, there are no existing tests for SIRS. Thus we cannot compare our proposed tests to any alternatives. Current bacteriologic diagnostic techniques for the demonstration of sepsis however, depend upon archaic bacterial culturing techniques which are slow (2-3 days) cumbersome and very expensive. Moreover they require specialized facilities and precautions. We expect that early Sepsis/SIRS differentiation by PCR will allow such culture techniques to be used much less frequently and for specialized indications.

2 Materials and Methods

2.1 SPE Tip Manufacturing Protocol

Materials Pipette Tip

- Thermo Scientific Finntip Pipette Tips 250 Universal, Cat.no. 9400-260
- O-rings Parker Seals, Cat.no. 2-006

Materials SPE

- Silica microspheres with an average diameter of 0.7 µm, Polysciences, Inc. (Warrington, PA)
- Butyl methacrylate (99%, BuMA), Sigma-Aldrich (St. Louis, MO)
- Ethylene dimethacrylate (98%, EDMA), Sigma-Aldrich (St. Louis, MO)
- 1-Dodecanol (98%), Sigma-Aldrich (St. Louis, MO)
- Cyclohexanol (99%), Sigma-Aldrich (St. Louis, MO)
- 2,2-dimethoxy-2-phenylacetophenone (99%, DMPAP), Sigma-Aldrich (St. Louis, MO)

All chemicals were used as received except for the monomer solutions, which were purified with a molecular sieve, also from Sigma-Aldrich to remove inhibitors.

Safety

- Work inside fume hood, especially when using compressed air
- If ever handling outside fume hood, wear Organic-vapor mask
- Dispose of all wastes in hazardous material jars

Microsphere preparation

- Put up to 1000 µL of silica microsphere suspension in an Eppendorf tube; make multiple tubes if necessary.
- Centrifuge at 2500 g for 10 min
- Decant supernatant
- Heat at 85°C until silica dried to a hard pill (45 min)
- Break up pill into a powder

Monolilth preparation

- Thaw Cyclohexanol and 1-Dodecanol in warm tap water
- Work inside fume hood!
- Melt DMPAP in microcentrifuge tube in block at 85°C
- Pipette BuMA, EDMA, Cyclohexanol, and 1-Dodecanol into warm batter tube.
- Add silica microspheres to batter tube (must be done after all other ingredients are mixed). Suspend by sonicating for 2-3 minutes, holding the tube near the top of the liquid – almost all (~90%) of the silica microsphere would go into suspension, leaving little behind.
- Pipette melted DMPAP into monolith batter tube. Vortex and load onto pipette tips

SPE batter proportions (AB)

BUMA	24
EDMA	16
1-Dodec	42
Cyclo	18
DMPAP	0.4
Silica susp	15

SPE batter recipe (SPE 100)
Yield: 4 *mL*

BUMA	960.0
EDMA	640.0
1-Dodec	1680.0
Cyclo	720.0
DMPAP	16.0
Silica susp (wet)	4000.0

Table 1: SPE batter recipe (SPE 100)

Pipette tip filling

- Agitate batter to disperse particles in SPE 100 batter
- Fill tips (50 µL)
- Pipette volume of batter into Finntip 250 tip. Pipette quickly to avoid bubbles
- Place pipette tips into UV oven, with tips angled down (Figure 1)
- UV cure for 15 min
- Rotate pipette tips by 180^{O} so that the bottom part is getting exposed to the UV

- UV cure for 15 min
- Add O-ring to pipette tip and load into aluminum bar (Figure 2)
- Load bar(s) into frame of Air pressure machine (Blackbird)
- Pipette 200 µL methanol into each pipette tip
- Start rinse at 50 psi until all methanol has dispensed
- Pipette 200 µL ethanol (70%) into each pipette tip
- Start rinse at 50 psi until all ethanol has dispensed
- Store cleaned pipette tips upright in original pipette box. The porous polymer doesn't form strong covalent bonds to the pipette surface. Therefore the porous polymer might detach from the pipette tip if not stored upright and handled with care.
- **Running samples at high pressure requires a de-pressuring step before the next loading step** (i.e. let the pressure settle to the surrounding atmospheric pressure before opening loading station)! Otherwise, if the pressure drop is too sudden, cracks might form in the polymer.

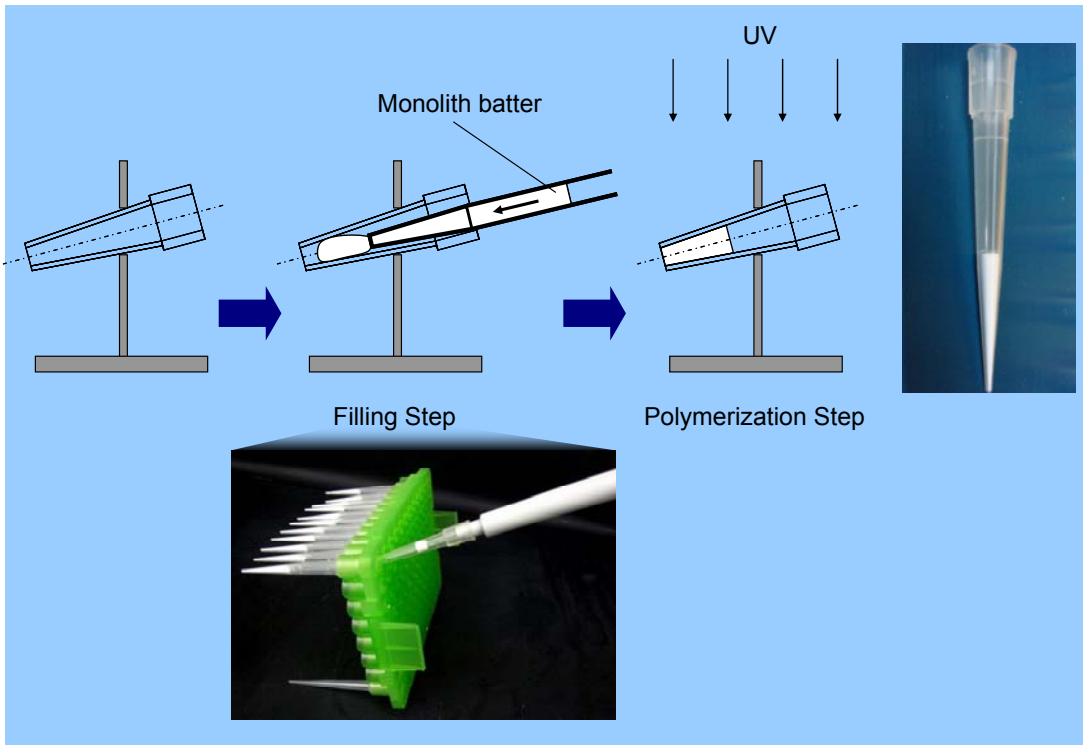

Figure 1: **Filling of pipette tip with monolith batter**

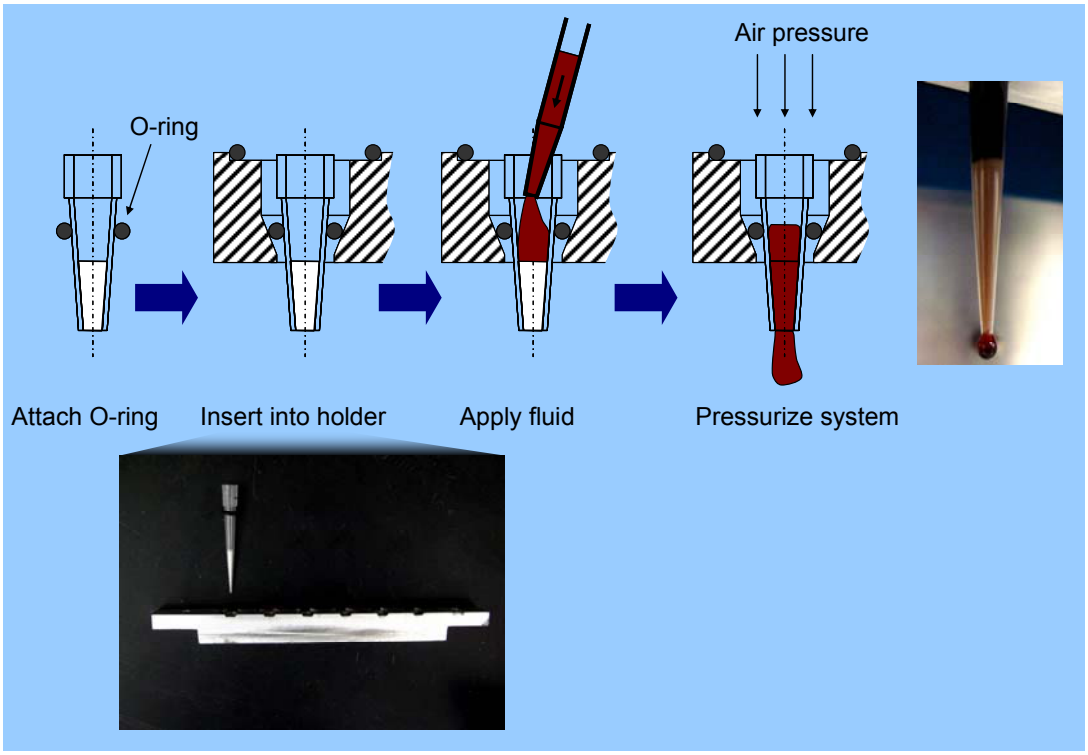

Figure 2: **Inserting pipette tip into holder and applying fluid**

2.2 DNA Extraction Procedures

2.2.1 mtDNA Extraction with QIAamp DNA Blood Mini Kit

For the extraction procedure, the protocol provided within the “QIAamp DNA Mini and Blood Mini Handbook 11/2007” was used. All chemicals and extraction devices used are from the QIAamp DNA Blood Mini Kit (50).

Materials

- mtDNA […]
- QIAamp DNA Blood Mini Kit (50), Qiagen, Cat.no. 51104
- Human serum, Sigma H4522
- Pooled human whole blood, Golden West Biologicals Inc., Cat.no. SD1030

Samples

- 100 ng (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 0.1 pg) of mtDNA in 200 µL human plasma or human blood respectively

Control

- 0 ng of mtDNA in 200 µL human plasma or human blood respectively

Procedures

- Pipet 20 µL QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube
- Add 200 µL sample to the microcentrifuge tube
- Add 200 µL Buffer AL (from QIAamp Kit) to the sample. Mix by pulse-vortexing for 15 s
- Incubate at 56°C for 10 min
- Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid
- Add 200 µL ethanol (100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid

- Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim, Figure 3 and Figure 4. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate

- Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided in kit), and discard the collection tube containing the filtrate

- Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min

- Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover

- Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 60 µL AE Buffer. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min

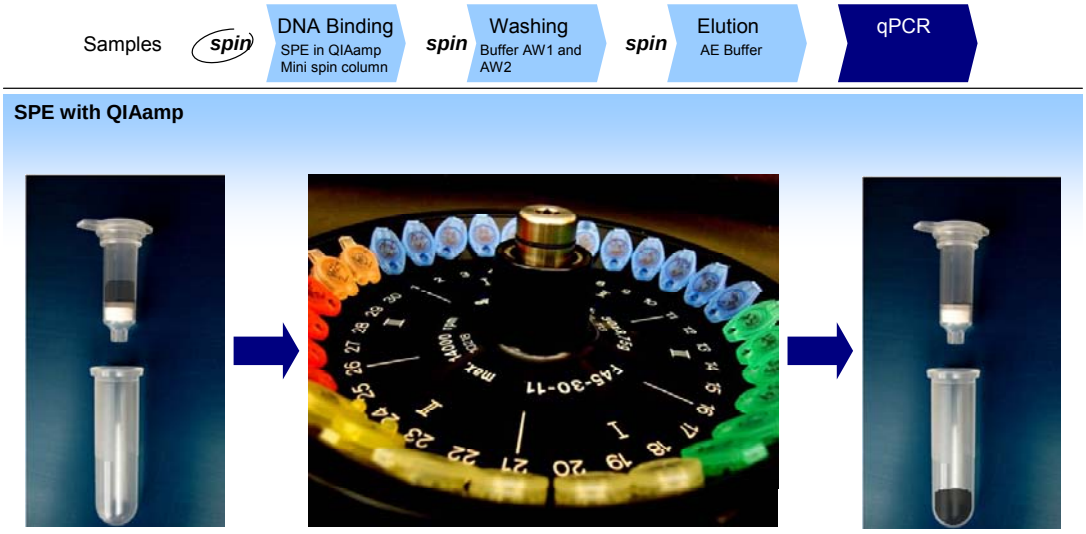

Figure 3: **Process Steps for Solid Phase Extraction (SPE) with QIAamp Mini Spin column**

Figure 4: **Scanning electron microscope photography of spin column**

2.2.2 mtDNA Extraction with SPE pipette tips

Materials

- mtDNA […]
- Pipette tips filled with SPE (see Chapter 2.1)
- Buffers from QIAamp DNA Blood Mini Kit (50), Qiagen, Cat.no. 51104
- Human serum, Sigma H4522
- Pooled human whole blood, Golden West Biologicals Inc., Cat.no. SD1030

Samples

- 100 ng (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 0.1 pg) of mtDNA in 64.5 µL human plasma or human blood respectively

Control

- 0 ng of mtDNA in 64.5 µL human plasma or human blood respectively

Procedures

- Pipet 6.45 µL QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml micro-centrifuge tube.
- Add 64.5 µL sample to the microcentrifuge tube
- Add 64.5 µL Buffer AL to the sample. Mix by pulse-vortexing for 15 s
- Incubate at 56°C for 10 min
- Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid
- Add 64.5 µL ethanol (100%) and mix by pulse-vortexing for 15 s in 1.5 ml microcentrifuge tube. After mixing, briefly centrifuge to remove drops from the inside of the tube's lid
- Apply the mixture to SPE columns (Figure 5 and Figure 6) and pressurize system to push mixture through PPM (100 psi)
- Add 160 µL Buffer AW1 and apply air pressure (100 psi)
- Add 160 µL Buffer AW2 and apply air pressure (100 psi)

- Apply pressure for 3 min to dry SPE columns (100 psi)
- Add 60 µL AE Buffer. Incubate at room temperature for 1 min, and then apply air pressure (50 psi)

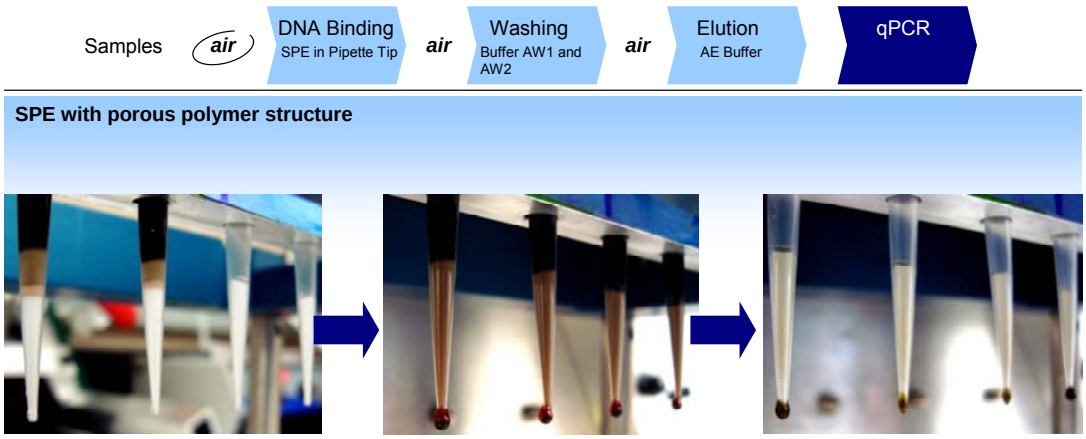

Figure 5: **Process Steps for Solid Phase Extraction (SPE) with porous polymer structure**

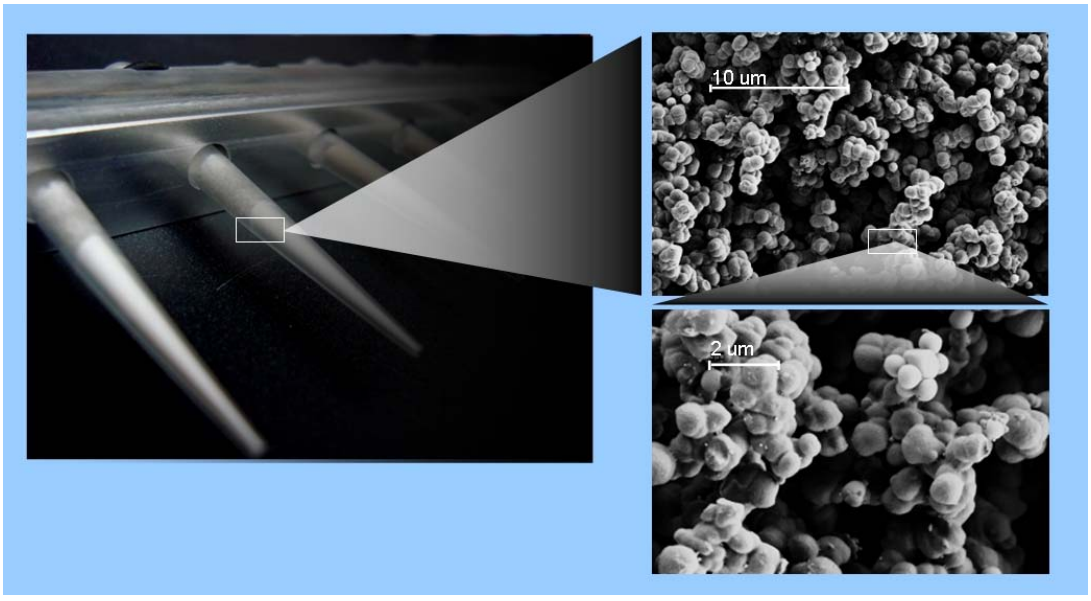

Figure 6: **Scanning electron microscope photography of porous polymer structure**

2.2.3 Growing E. Coli Cells for Experiments

Materials

- E. Coli GFP Cells
- LB Broth
- E. Coli GFP Cells

Procedures

See Madhumita Mahalanabis Paper (Lab Chip, 2009 DOI: 10.1039/b905065p, Cell lysis and DNA extraction of gram-positive and gram-negative bacteria from whole blood in a disposable microfluidic chip) for additional information.

- If plates are older than 2 months, the E. Coli may not grow. Make a new plate then by using purchased E. Coli cultures. Pre-warm LB plate at 37°C for 30 min) and incubate at 37°C for 16-18hrs. IMPORTANT: Put the bacterial plates back in the fridge after wrapping in parafilm. If the plate is not re-wrapped and back in the fridge the cells will die.
- Use 15 ml Falcon culture tubes (transparent caps). Take 3 tubes. Inoculate 2 tubes of 3 ml LB broth cultures of E. coli GFP and 1 for a negative LB only control.
- Use LB broth from the 4C deli fridge. Open the bottle only under bunsen burner flame, flaming the bottle opening every time you open the bottle and every time before replacing the lid (so twice every time you go into the bottle).
- Inoculation: From the LB agar streak plate -- pick 1 colony with either a sterile toothpick (on shelf above bench) or with sterile 10ul micropipette tip. If using a tip, use steel forceps; dunk the tip of the forceps in 95% ethanol and flame to ignite the ethanol. This sterilizes the forceps. Then grab the tip, touch it to the colony, and release the tip into the top of the culture tube. Do not touch the sides of the tube with the forceps or your hand for sterility.
- IMPORTANT: Do the same procedure for the negative tube – add 3 ml of broth and add a sterile pipette tip or toothpick (with no E. coli). This tube serves as a control for your sterile technique.
- IMPORTANT: Loosely cap the tubes -- they have 2 stops. The first allows for air exchange which is critical, use this. You should be able to pull up on the cap gently without it coming off. The second closes the cap fully and seals the tube. Don't seal the tubes completely.

- Incubate the 3 tubes at a 45 degree angle roughly at 37C in a shaking incubator. We have one in 709 on the bench next to the large eppendorf centrifuge. You can use this by placing your tubes in a small rack and fitting the rack inside the elastic clamps. Turn on and set at 37°C, 225-250 RPM speed. Grow for 16 hours. If grows longer then the DNA yield will be reduced due to cell lysis in the broth

- IMPORTANT: After exactly 16 hrs store at 4°C in the fridge for up to 2 hrs unless using it right away. For longer storage pipette 1 ml volumes of the broth into sterile 1.7 ml centrifuge tubes and pellet the cells. Pellet at 8000 RPM for 10 min. Remove all supernatant without disturbing cell pellet. Store cell pellets at -20°C freezer for up to 1 month. Label tube as 1 ml E. coli GFP pellet, date, initials

- These cell pellets can be thawed, re-suspended in 1 ml broth by vigorous vortexing at maximum speed until the pellet disappears, diluted in a serial 10-fold dilution for the desired cell concentration, re-pelleted, suspended in the lysis buffer/broth mix and then extracted

- If ready to do the DNA extraction right away after the culture has grown 16hrs, you can skip the initial cell pelleting and freezing step

- The number of cells is approx. 1X10^9 cells/ml after 16 hrs in LB broth at 37°C. For exact number see the methods section of LOAC paper for E. coli.

2.2.4 E. Coli Cell Lysis and DNA Extraction with QIAamp DNA Blood Mini Kit

For the extraction procedure, the protocol provided within the "QIAamp DNA Mini and Blood Mini Handbook 11/2007" was used. All chemicals and extraction devices used are from the QIAamp DNA Blood Mini Kit (50).

Materials

- E. Coli GFP Cells
- QIAamp DNA Blood Mini Kit (50), Qiagen, Cat.no. 51104
- Pooled human whole blood, Golden West Biologicals Inc., Cat.no. SD1030

Samples

- 10^4, 10^3, 10^2, E. Coli Cells in 200 µL human blood

Control

- 0 E. Coli Cells in 200 µL human blood

Procedures

- Pipet 20 µL QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube
- Add 200 µL sample to the microcentrifuge tube
- Add 200 µL Buffer AL (from QIAamp Kit) to the sample. Mix by pulse-vortexing for 15 s
- Incubate at 56°C for 10 min
- Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid
- Add 200 µL ethanol (100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid
- Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate

- Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided in kit), and discard the collection tube containing the filtrate

- Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min

- Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover

- Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 60 µL AE Buffer. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min

2.2.5 E. Coli Cell Lysis and DNA Extraction with SPE pipette tips

Materials

- Pipette tips filled with SPE (see Chapter 2.1)
- Buffers from QIAamp DNA Blood Mini Kit (50), Qiagen, Cat.no. 51104
- Pooled human whole blood, Golden West Biologicals Inc., Cat.no. SD1030

Samples

- 10^4, 10^3, 10^2 E. Coli GFP Cells in 64.5 µL human blood

Control

- 0 E. Coli Cells in 64.5 µL human blood

Procedures

- Pipet 6.45 µL QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml micro-centrifuge tube.
- Add 64.5 µL sample to the microcentrifuge tube
- Add 64.5 µL Buffer AL to the sample. Mix by pulse-vortexing for 15 s
- Incubate at 56°C for 10 min
- Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid
- Add 64.5 µL ethanol (100%) and mix by pulse-vortexing for 15 s in 1.5 ml microcentrifuge tube. After mixing, briefly centrifuge to remove drops from the inside of the tube's lid
- Apply the mixture to SPE columns and pressurize system to push mixture through PPM (100 psi)
- Add 160 µL Buffer AW1 and apply air pressure (100 psi)
- Add 160 µL Buffer AW2 and apply air pressure (100 psi)
- Apply pressure for 3 min to dry SPE columns (100 psi)
- Add 60 µL AE Buffer. Incubate at room temperature for 1 min, and then apply air pressure (50 psi)

2.3 PCR

Materials

- AE Buffer from QIAamp DNA Blood Mini Kit (50), Qiagen, Cat.no. 51104
- SYBR Green PCR Master Mix, Applied Biosystems, Cat.no. 4309155
- Primers for mtDNA
  Cyt-B forward: 5'-TGAGGGGCTTCTCAGTAGA-3'
  Cyt-B reverse: 5'-GGAGGTGAACGATTGCTAGG-3'
- Primers for E. Coli
  16S-1 forward: 5'-TGTAGCGGTGAAATGCGTAGA-3'
  16S-1 reverse: 5'-CCAGGGTATCTAATCCTGTTTG-3'

qPCR mix

For mtDNA

- 1 µL of 20 uM Cyt-B forward1 µL of 20 uM Cyt-B reverse
- 12.5 µL of SYBR Green PCR Master Mix
- 5.5 µL of nuclease free water
- 5 µL of sample

For E. Coli

- 1 µL of 20 uM 16S-1 forward
- 1 µL of 20 uM 16S-1 reverse
- 12.5 µL of SYBR Green PCR Master Mix
- 5.5 µL of nuclease free water
- 5 µL of sample

Control

- AE Buffer (negative control)
- Nuclease free water (negative control)
- For mtDNA Samples
  Dilutions of x ng mtDNA in 60 µL nuclease free water:
  x = 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg

Temperature Profile

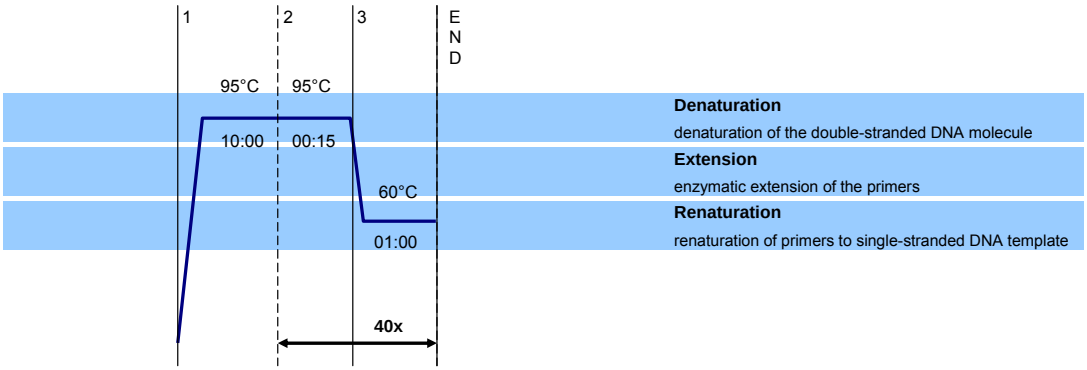

Figure 7: **Temperature Profile for qPCR**

3 Results and Discussion

3.1 mtDNA Extraction

[…]

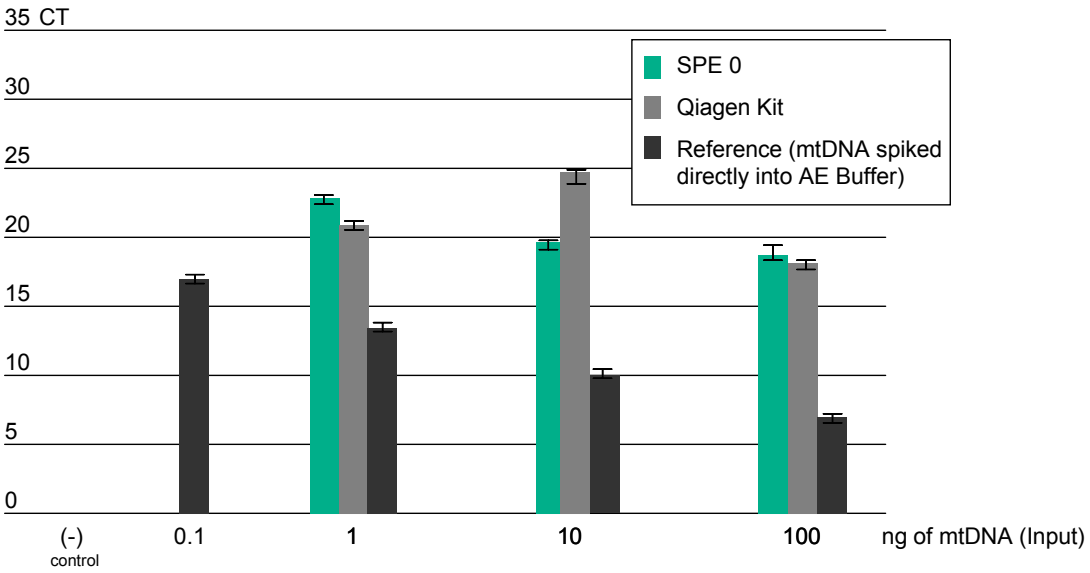

Figure 8: **qPCR results for mtDNA spiked into human serum**

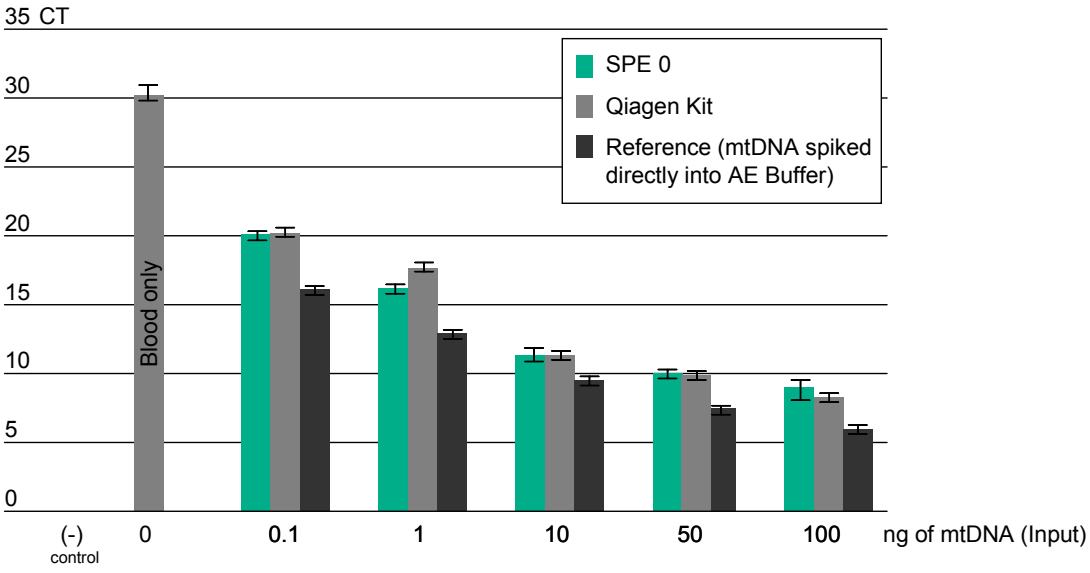

Figure 9: **qPCR results for mtDNA spiked into pooled human blood (100 pg to 100 ng mtDNA)**

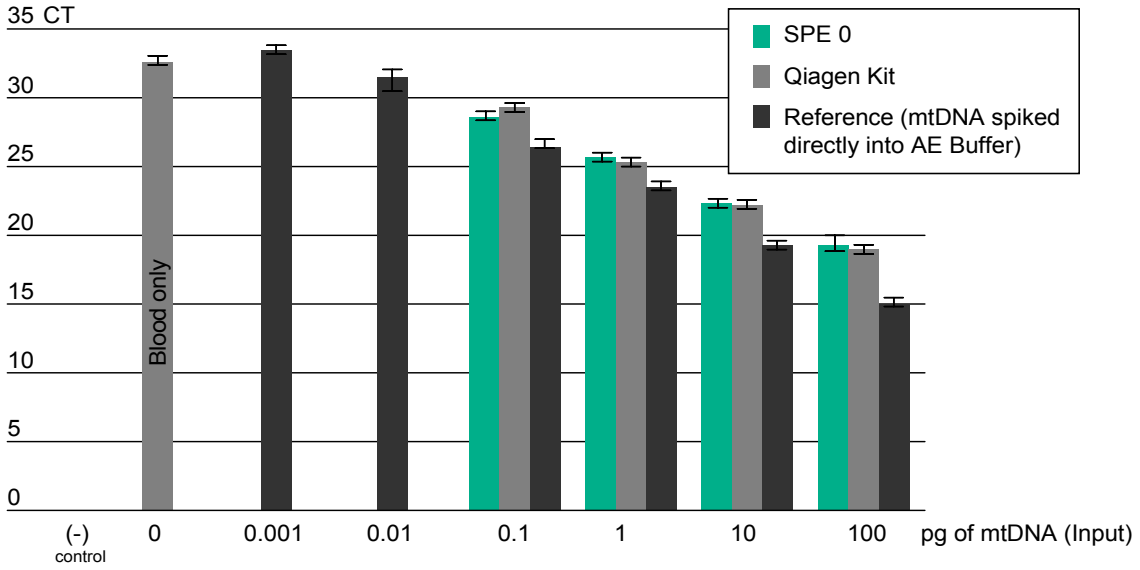

Figure 10: **qPCR results for mtDNA spiked into pooled human blood (100 fg to 100 pg mtDNA)**

3.2 E. Coli Cell Lysis and DNA Extraction

[…]

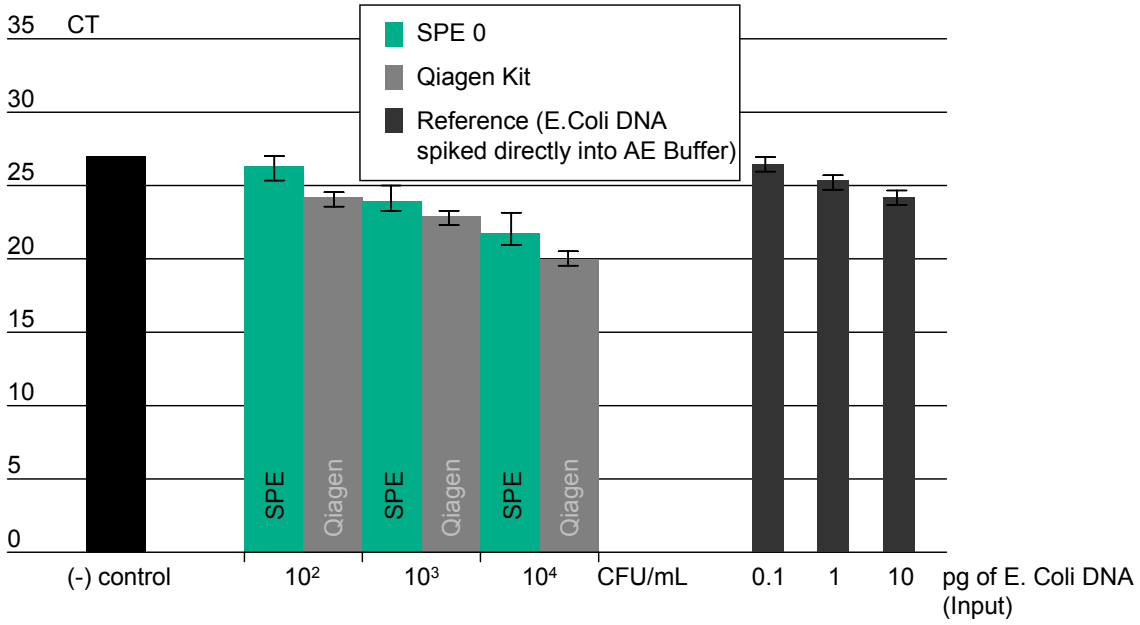

Figure 11: **qPCR results for E. Coli cells spiked into pooled human blood**

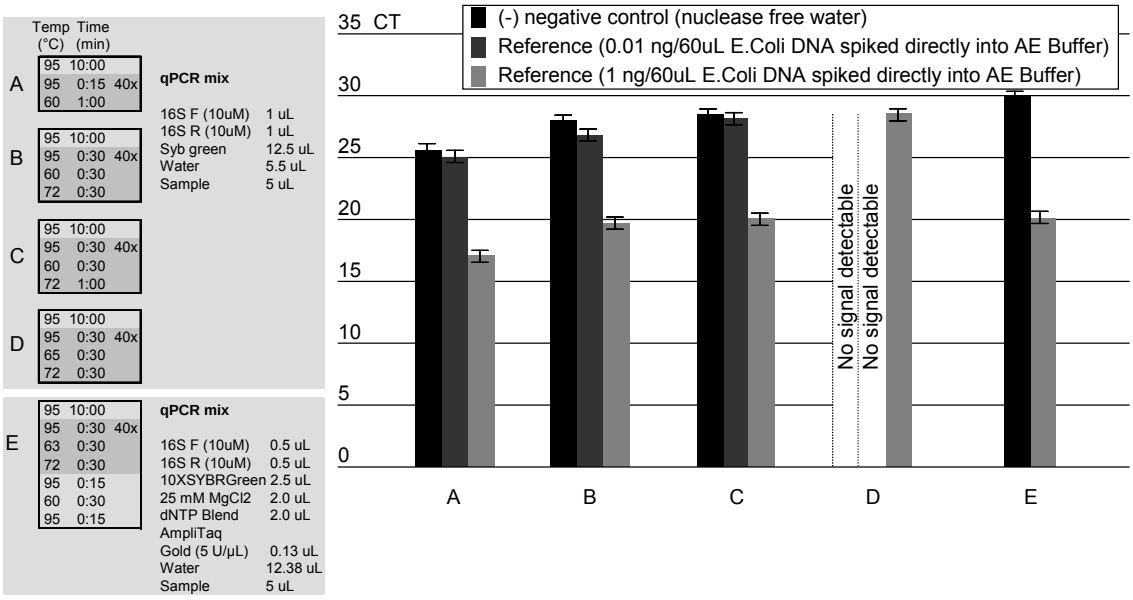

Figure 12: qPCR results for E. Coli DNA spiked into AE Buffer (Temp. profile variation)

4 Conclusions and Future Work

[…]

5 Literature

[…]

Appendix

[…]

MITOCHONDRIAL DAMPS RELEASED BY ABDOMINAL TRAUMA SUPPRESS PULMONARY IMMUNE RESPONSES

Abstract No:

432

Abstract Submission Type:

EAST Member Submission

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Objectives:

Historically, fever, pneumonia and sepsis after chest trauma is ascribed to pain, splinting and poor pulmonary toilet. But no evidence supports those assertions and no biologic mechanisms have been advanced to explain these associations. Our studies have shown injured tissues release mitochondrial debris that attracts neutrophils (PMN). Thus we hypothesized mitochondria (MT) released by injured, dying tissue could divert neutrophils from the lung, leaving it susceptible to bacterial invasion.

Methods:

Anesthetized rats (6-10/group) underwent chest percussion to induce pulmonary contusion (PC). To model MT release from liver injury, some rats had sonicated MT isolated from rat liver (equivalent to MT from 5% liver necrosis) injected into the peritoneal cavity. At 16h bronchoalveolar and peritoneal lavages were performed. Lavage fluids (BALF, PLF) were assayed for PMN count, albumin, IL-β and CINC.

Results:

PC caused a 50-fold increase in BALF neutrophils (Fig 1) and tripled lung albumin leak (Fig 2). Peritoneal MT had no direct effect on lung PMN or leak, but increased peritoneal IL-β and caused marked influx of PMN and albumin into the peritoneum. In rats undergoing PC, additional injection of MT into the peritoneum markedly decreased BALF PMNs ($P<0.001$, Fig 1) and albumin leak ($P<0.002$, Fig 2).

Conclusions:

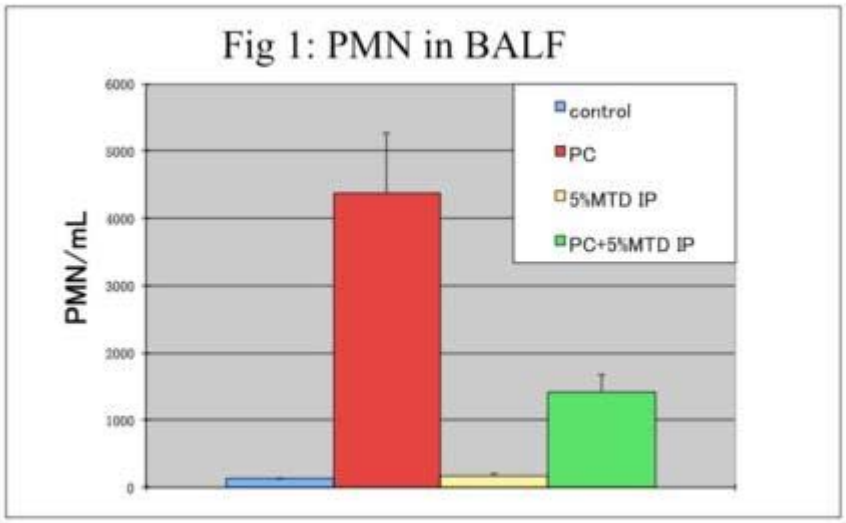
Rather than acting as a 'second hit' to induce PMN-mediated lung injury, MT debris acts as a chemoattractant, diverting PMN away from lung injury to systemic sites of injury. This may diminish acute lung injury but it is expected to make the lung susceptible to infection. This novel paradigm provides a direct mechanistic model of the relationship between systemic blunt tissue injury, pneumonia and sepsis that can now be studied and used to improve care and trauma outcomes.

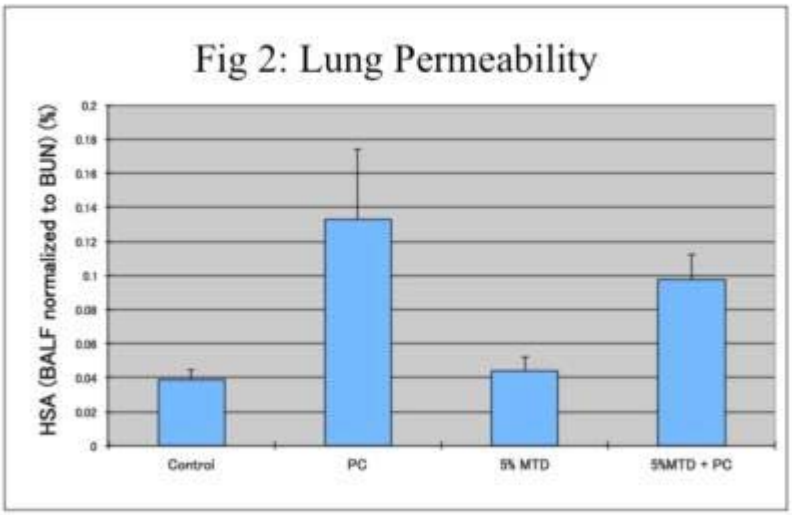
Abstract Overall Category (Required):

Basic Science

Presenting Author Demographics:

Trauma/Acute Care Surgeon





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