

Mitochondrial DNA Stimulates TLR9-Dependent Neutrophil Extracellular Trap Formation in Primary Graft Dysfunction

Beñat Mallavia, Fengchun Liu, Emma Lefrançais, Simon Cleary, Nicholas Kwaan, Jennifer Tian, Melia Magnen, David Sayah, Allison Soong, Joy Chen,

et al.

► To cite this version:

Beñat Mallavia, Fengchun Liu, Emma Lefrançais, Simon Cleary, Nicholas Kwaan, et al.. Mitochondrial DNA Stimulates TLR9-Dependent Neutrophil Extracellular Trap Formation in Primary Graft Dysfunction. American Journal of Respiratory Cell and Molecular Biology, 2020, 62 (3), pp.364-372. 10.1165/rcmb.2019-0140OC . hal-02997951

HAL Id: hal-02997951 https://cnrs.hal.science/hal-02997951

Submitted on 10 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Mitochondrial DNA Stimulates TLR9-Dependent NET Formation in Primary Graft Dysfunction

Beñat Mallavia¹, Fengchun Liu¹, Emma Lefrançais¹, Simon J. Cleary¹, Nicholas Kwaan¹, Jennifer J. Tian¹, Melia Magnen¹, David M. Sayah⁴, Allison Soong¹, Joy Chen³, Rajan Saggar⁴, Michael Y. Shino⁴, David J. Ross⁴, Ariss Derhovanessian⁴, Joseph P. Lynch III⁴, Abbas Ardehali⁵, S. Sam Weigt⁴, John A. Belperio⁴, Steven R. Hays¹, Jeffrey A. Golden¹, Lorriana E. Leard¹, Rupal J. Shah¹, Mary Ellen Kleinhenz¹, Aida Venado¹, Jasleen Kukreja³, Jonathan P. Singer¹, Mark R. Looney^{1,2}

¹Departments of Medicine, ²Laboratory Medicine, and ³Surgery, University of California, San Francisco; San Francisco, CA, USA.

⁴Departments of Medicine and ⁵Surgery, University of California, Los Angeles; Los Angeles, CA, USA.

Correspondence to: Mark R. Looney, M.D. 513 Parnassus Avenue San Francisco, CA 94143-0130 Tel: (415) 476-9563 mark.looney@ucsf.edu

Author contributions

BM and MRL designed the research studies. BM, FL, EL, NK, SJC, and MM conducted the experiments. BM and SJC acquired the data. BM, FL, JJT, DMS, AS, JC, RS, MYS, DJR, AD, JPL, AA, SSW, JAP, SRH, JAG, LEL, RJS, MEK, AV, JK, JPS, and MRL collected or processed the human biospecimens. BM, EL, NK, SJC, MM, JPS, and MRL analyzed the data. BM and MRL wrote the manuscript.

Funding

This work is supported by NIH grants R01 HL130324 and R01 Al125445 (to M.R. Looney), RO1 HL134851 (to J.P. Singer), and the Nina Ireland Program in Lung Health (to M.R. Looney).

Running title: mtDNA stimulates TLR9-dependent NETs in PGDSubject category: 16.4 (Transplantation: Experimental)Word count: 3307

This article has an online data supplement, which is accessible from this issue's table of content online at <u>www.atsjournals.org</u>.

Abstract

The immune system is designed to robustly respond to pathogenic stimuli but to be tolerant to endogenous ligands to not trigger autoimmunity. Here, we studied an endogenous damage-associated molecular pattern, mitochondrial DNA (mtDNA), during primary graft dysfunction (PGD) after lung transplantation. We hypothesized that cellfree mtDNA released during lung ischemia-reperfusion triggers neutrophil extracellular trap (NET) formation via TLR9 signaling. We found that mtDNA increases in the bronchoalveolar lavage fluid of experimental PGD (prolonged cold ischemia followed by orthotopic lung transplantation) and not in control transplants with minimal warm ischemia. The adoptive transfer of mtDNA into the minimal warm ischemia graft immediately prior to lung anastomosis induces NET formation and lung injury. TLR9 deficiency in neutrophils prevents mtDNA-induced NETs, and TLR9 deficiency in either the lung donor or recipient decreases NET formation and lung injury in the PGD model. Compared to human lung transplant recipients without PGD, severe PGD was associated with high levels of bronchoalveolar lavage mtDNA and NETs, with evidence of relative deficiency in DNasel. We conclude that mtDNA released during lung ischemia-reperfusion triggers TLR9-dependent NET formation and drives lung injury. In PGD, DNasel therapy has a potential dual benefit of neutralizing a major NET trigger (mtDNA) in addition to dismantling pathogenic NETs.

Abstract word count: 204

Key words: primary graft dysfunction, mitochondrial DNA, neutrophil extracellular traps, TLR9

Introduction

Solid organ transplantation is a life-saving approach for thousands of patients with endstage organ dysfunction. Major advances have been made in perioperative management and in the treatment of acute and chronic rejection, however many challenges remain to improve clinical outcomes. In the United States, more than 2300 lung transplant surgeries are performed each year for a variety of end-stage lung diseases (1). The major early complication after transplantation is primary graft dysfunction (PGD), which occurs within the first 72 hours after surgery, drives early morbidity and mortality, and is a risk factor for chronic lung allograft dysfunction (2-4). PGD is induced by ischemia-reperfusion lung injury and shares many similarities to the adult respiratory distress syndrome (ARDS) (5). Indeed, it is defined by similar clinical criteria and the grading system is based on the severity of the oxygenation defect (6). The pathophysiology of PGD is complex, but innate immune activation, perhaps excessive, is an integral feature. Neutrophils prominently accumulate in the lungs during PGD and their activation is likely responsible for the breakdown in the lung barrier and alveolar flooding (7).

We have previously reported that neutrophil extracellular traps (NETs) are produced in abundance in a mouse model of PGD based on prolonged donor lung cold ischemia and in humans with severe PGD (8). While being potentially protective in infectious conditions through microbial containment, NETs have been implicated in a variety of non-infectious diseases where they have generally been responsible for bystander tissue injury (9, 10). In our model of PGD in mice, for example, the application of intrabronchial DNasel therapy at the time of transplantation significantly reduced lung inflammation and improved lung function (8). In a mouse model of bacterial pneumonia, an important cause of ARDS, DNasel therapy also reduced lung injury and improved survival, while not impairing bacterial containment (11).

The trigger for NET production in PGD is unknown. Here, we hypothesized that NET formation could be induced by damage-associated molecular patterns (DAMPs) released from injured cells during ischemia-reperfusion. Potential DAMPs previously implicated in inflammatory states, and that could be involved in the pathogenesis of PGD, include release products from damaged mitochondria (DNA with CpG motifs, formylated peptides), HMGB1, and ATP among others (12). Disrupted mitochondria are implicated in trauma-induced organ dysfunction, including lung injury (13), and we therefore focused our studies on mitochondrial DNA (mtDNA) and the potential for NET induction via endosomal TLR9 activation.

Methods

Additional detail on the mouse surgical procedures, lung intravital microscopy, NETs measurements, and human biospecimens are provided in the online supplement.

Mice

8 to 12 week-old male mice housed under specific pathogen-free conditions were used for all experiments. C57BL/6J and *mTmG* mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *TLR9*^{-/-} mice were obtained from Charles Kim (UCSF). *PAD4*^{-/-} mice were obtained from Kerri Mowen (The Scripps Research Institute, La Jolla, CA).

Orthotopic lung transplant model of PGD

Orthotopic left lung transplants were carried out using the specified mouse genotypes, as previously described (8). The prolonged cold ischemia (PCI) model of PGD was induced by storing the left donor lung in sterile gauze soaked in Perfadex at 4°C for 18 hours prior to transplantation into the recipient mouse with minimal warm ischemia (8). A second group of lung transplants with no cold ischemia and minimal warm ischemia (8). A second group of lung transplants with no cold ischemia and minimal warm ischemia (immediate transplantation) were included as surgical controls for the PCI transplants. In selected experiments, DNasel (2,000 units in 15 μ I diluent, Roche, Indianapolis, IN) vs. diluent alone (20 mM Tris-HCI, 1mM MgCl₂) were directly instilled into the donor lung bronchus prior to bronchial anastomosis. mtDNA was extracted from mouse liver and isolated using the XIT Mitochondrial DNA kit (G-Biosciences, St. Louis, MO). In selected experiments, mtDNA (50 μ g in 15 μ I diluent) vs. diluent alone were directly instilled into the donor lung bronchus prior to bronchial prior to bronchial DNA kit (G-Biosciences, St. Louis, MO). In selected experiments, mtDNA (50 μ g in 15 μ I diluent) vs. diluent alone were directly instilled into the donor lung bronchus prior to bronchial prior to bronchial DNA kit (G-Biosciences, St. Louis, MO). In

Lung intravital microscopy

A modified version (14) of the previously published method of stabilized lung intravital imaging (15) was used. *mTmG* donor and recipient mice were used in minimal warm ischemia and PCI transplant models. To stain extracellular DNA, SYTOX Green was instilled intratracheally (50 μ L of 1.0 mM solution) and intravenously (50 μ L of 0.5 mM solution).

mtDNA and TLR9 quantitative PCR

mtDNA was quantified in cell-free BAL (1 μ L) fluid samples (mouse and human) by amplification of the mitochondria-specific Cytochrome C Oxidase 1 gene using mouse or human-specific primer/probe sets (Applied Biosystems, Foster City, CA). TLR9 receptor expression was measured in bone marrow neutrophils from WT or *TLR9*-^{/-} mice using TLR9 primers and GAPDH primers (Taqman, Thermo Fisher Scientific, Waltham, MA).

In vitro NET generation and immunofluorescence

Neutrophils (5 x 10⁵) were plated and stimulated for 4 hours at 37°C and 5% CO₂ with 4 μ M ionomycin (Adipogen, San Diego, CA) or 100 μ g/mL of mtDNA or nuclear DNA. Nuclear DNA was isolated with the NucleoSpin DNA RapidLyse kit (Macherey-Nagel, Düren, Germany). In select experiments, mtDNA was pretreated with 70 U of DNasel (Roche) for 30 minutes at 37°C and then 5 minutes at 75°C to deactivate DNasel (16) prior to incubation with neutrophils.

NET and DNasel ELISAs

NETs in cell culture supernatants and mouse or human BAL/plasma samples were quantified using ELISAs as previously described (11). DNasel levels in human BAL/plasma were measured using a DNasel ELISA (MBS2024612, MyBiosurce, San Diego, CA) following the manufacturer's instructions.

Acute lung injury measurements

BAL total protein and white bloods cells and differential were measured as previously described (8, 11).

Arterial blood gas measurements

Blood was collected from the left ventricle into heparinized syringes for arterial blood gas measurements. Arterial blood gases were measured using a VetScan i-STAT 1 handheld analyzer and CG4+ cartridges (Abaxis, Union City, CA).

Human biospecimens

BAL and plasma samples were prospectively collected from two independent cohorts of subjects (UCSF and UCLA) on the morning of the first post-operative day following lung transplant surgery from April 2016 to August 2017. BAL was obtained as part of routine clinical bronchoscopy by the subjects' lung transplant clinician. PGD was defined and scored per international guidelines based on chest radiography and oxygenation (17).

Statistics

All in vivo and in vitro experiments were repeated a minimum of three independent times. Unless otherwise noted, all mouse data are presented as mean \pm SEM and human subject data are presented as min-to-max whiskers and box plots showing the median and interquartile ranges. A *P* value less than or equal to 0.05 was considered significant. All statistical analyses were performed using GraphPad Prism 7.0

(GraphPad Software, Inc. San Diego, CA) and STATA v15.1 (StataCorp, College Station, TX).

Study approval

All experiments were approved by the Institutional Animal Care and Use Committee (UCSF) and the Committee on Human Research (UCSF and UCLA). Written informed consent was obtained from participants prior to inclusion in the study.

Results

mtDNA is released during experimental PGD and is completely degraded by DNasel. We used orthotopic lung transplantation (OLT) in syngeneic mice and prolonged cold ischemia (PCI) of the donor lung (8) to test for the release of extracellular DNA (mtDNA) or NETs) during organ reperfusion. Using intravital lung microscopy (18), we observed increased extracellular DNA in alveoli during graft reperfusion in mice transplanted with PCI compared to transplants with minimal warm ischemia (Figures 1A-B and Supplemental Movies 1 and 2). We reasoned that the intra-alveolar DNA could be derived from NETs, as previously described (8), or released from injured lung cells during ischemia-reperfusion. We measured mtDNA accumulation in the bronchoalveolar lavage (BAL) of transplanted mice by quantitative PCR using a mitochondrial-specific probe that binds to cytochrome C oxidase 1. Lungs transplanted with PCI produced higher mtDNA levels compared to mice transplanted with lungs with minimal warm ischemia or non-transplanted control BAL (Figure 1C). We instilled DNasel intrabronchially immediately prior to lung anastomosis, which completely degraded mtDNA in the PCI model compared to the diluent control (Figure 1D).

Page 10 of 50

Intrabronchial administration of mtDNA prior to lung anastomosis induces NET formation and lung injury. To determine if mtDNA is sufficient to cause lung injury in the setting of lung transplantation, we tested the adoptive transfer of mtDNA into the donor lung immediately before lung anastomosis in the minimal warm ischemia transplant model, which does not have elevated levels of BAL mtDNA during reperfusion (Figure 1C). As expected, mice instilled with intrabronchial mtDNA had higher values of BAL mtDNA at 8 hours post-reperfusion compared to diluent controls (Figure 2A). The mtDNA group also had higher levels of BAL NETs (CitH3-DNA complexes; Figure 2B), increased protein permeability (Figure 2C), increased neutrophilic inflammation (Figures 2D-E), and decreased oxygenation (Figure 2F), all indicating that mtDNA is sufficient to induce NET formation and lung injury after lung transplantation.

mtDNA induces TLR9-dependent NET formation. To further study the possible effect of cell-free mtDNA on NET induction, mouse neutrophils were isolated from bone marrow and stimulated with ionomycin (positive control) or mtDNA isolated from mouse liver. NETs were detected using a neutrophil elastase-DNA ELISA in cell-free supernatants and with immunofluorescence (8). Ionomycin and mtDNA-stimulated neutrophils both efficiently generated NETs (Figures 3A and 3C). Incubation of mtDNA with DNasel prior to incubation with neutrophils failed to induce NETs indicating that mtDNA was responsible for NET formation and not other products potentially contaminating the mtDNA during isolation and purification (Figure 3A). As an additional control, nuclear DNA was isolated from mouse liver and failed to induce NETs (Figure 3A).

We next tested the molecular pathway by which mtDNA induces NETs. Endosomal TLR9 recognizes CpG motifs in mtDNA resulting in activation of NF-κB and downstream

inflammatory pathways (13). Using quantitative PCR, we confirmed that TLR9 mRNA is present in isolated neutrophils (Supplemental Figure 1). mtDNA failed to induce NETs in *TLR9*-/- neutrophils while ionomycin stimulation efficiently induced NETs in these cells (Figure 3B and 3C). mtDNA incubated with DNasel, or nuclear DNA, did not induce NET production in *TLR9*-/- neutrophils (Figure 3B). We also tested a TLR9-specific agonist (ODN-1668), which induced NETs in WT but not *TLR9*-/- neutrophils (Figure 3C).

TLR9 deficiency decreases NET production and lung injury. To test for the role of the mtDNA-TLR9 axis in the pathogenesis of PGD, we produced lung transplant chimeras in the PCI model in the following groups: WT donor lung transplanted into WT recipient, *TLR9*-/- donor lung transplanted into *TLR9*-/- recipient, WT donor lung transplanted into *TLR9*-/- recipient, or *TLR9*-/- donor lung transplanted into WT recipient. Mice were euthanized at 8 hours post-transplantation and lung injury was measured. Compared to WT----WT transplants, any other combination of lung transplantation that incorporated TLR9 deficiency yielded decreased alveolar NETs (Figure 4A), decreased lung permeability (Figure 4B), decreased neutrophilic inflammation (Figure 4C and 4D), and improved lung function as measured by arterial oxygenation (Figure 4E). These data demonstrate that TLR9 is stimulated in both lung native cells and infiltrating immune cells in experimental PGD to generate NETs and produce lung injury.

Peptidyl arginine deiminase 4 deficiency reduces citrullinated NETs and limits the extent of lung injury in PGD. Peptidyl arginine deiminase 4 (PAD4) is a critical mediator of NET formation by citrullinating histones and thus facilitating chromatin decondensation during NET formation (19). NET generation is decreased in PAD4 deficient mice (20) as well as with the use of PAD4 specific inhibitors (21). To further study the relevance

Page 12 of 50

of NET-induced lung injury in PGD, we analyzed NET generation and lung injury parameters in the absence of PAD4. We first confirmed the lack of citrullinated NETs (CitH3-DNA complexes) in *PAD4*-/- neutrophils after mtDNA stimulation relative to WT neutrophils (Figure 5A). Next, we tested PAD4 deficient lung transplants versus WT control transplants in the PGD model. PAD4 deficient transplants yielded decreased BAL citrullinated NETs (Figure 5B), neutrophilic inflammation (Figure 5C and 5D), and a trend towards decreased lung permeability (Figure 5E). Arterial oxygenation was significantly improved in PAD4 deficient transplants (Figure 5F).

Severe PGD in humans is associated with increased BAL mtDNA and NET levels and *increased NET/DNasel ratios*. To verify our data in the experimental PGD model, we analyzed human BAL and plasma samples collected on the first post-operative day after lung transplantation at two medical centers. We grouped subjects into either no PGD at any time point (PGD 0/1 at 24, 48, and 72 hours post-transplant) or severe PGD (PGD 2/3) at each time point post-transplant. There were no significant differences in the demographics and clinical characteristics between the severe PGD and no PGD groups (Supplemental Table). We quantified total white blood cells, neutrophils, and monocytes in the BAL of these subjects, and although there was a trend towards higher neutrophil counts in patients with severe PGD, none of the three parameters were statistically different (Supplemental Figure 2A-C). We detected higher mtDNA levels in BAL fluid, but not plasma, in severe PGD compared to no PGD (Figure 6A-B). NET levels were also increased in the BAL of severe PGD compared to no PGD (Figure 6C-D). Although DNasel concentration was not significantly different between the two PGD groups (Figure 6E-F), there was a significant increase in the NETs to DNasel ratio in the BAL of severe PGD compared to no PGD (Figure 6G-F). Receiver operating characteristic curves generated for mtDNA, NETs, or NET/DNasel measurements early after transplantation showed that BAL NETs/DNasel was most associated with concurrent or subsequent severe PGD status (Supplemental Figure 3A-D). We also correlated BAL mtDNA content with the PaO₂/FiO₂ in the human cohort and our mouse experiments and observed a negative correlation between BAL mtDNA and PaO₂/FiO₂ (Human cohort correlation coefficient = -0.47, *p* = 0.0136; Mouse model correlation coefficient = -0.53, *p* = 0.0333).

Discussion

Ischemia-reperfusion injury is a complex process during which reduced ATP generation and increased reactive oxygen species can induce tissue injury and neutrophil accumulation/activation, leading to further injury (22). Neutrophil accumulation may lead to extracellular DNA trap formation that can perpetuate organ dysfunction by directly injuring endothelial and epithelial cells (9, 23). Previously, we described NET accumulation in experimental PGD and in humans with severe PGD and determined that dismantling extracellular chromatin with DNasel in mice significantly improved graft function (8). However, the mechanism by which NETs were produced was not identified.

We have now discovered that mtDNA, released during lung ischemia-reperfusion injury, accumulates in the alveolar compartment, also the site of injury in PGD. Increased mtDNA levels have been reported in a variety of pathologic conditions, both sterile and pathogen-induced, including sepsis, trauma, ARDS, and others (24-26). It is now clear that cell-free mtDNA is more than just a marker of cellular injury, but a potent inducer of

Page 14 of 50

inflammatory responses. CpG motifs within mtDNA are recognized by TLR9 present in a variety of immune and non-immune cells (27). mtDNA signals through TLR9 to induce NF-κB, MAPK, and other downstream inflammatory mediators (28, 29). TLR9, and other TLRs that recognize nucleic acids, initially traffic to the cell membrane, but perhaps to restrict access to nucleic acids, ultimately reside on endosomal membranes. For example, cell-surface overexpression of TLR7, which is normally endosomal, leads to systemic autoimmunity through self-recognition of nucleic acids (30). Recent data indicates that red blood cells retain signaling-incompetent TLR9 on the cell surface that can serve to scavenge endogenous TLR9 agonists such as mtDNA to limit inflammation (31).

The administration of mtDNA to lung grafts immediately prior to transplantation was sufficient to induce lung injury and NET formation. mtDNA can trigger NET formation via TLR9 expressed in neutrophils, which concurs with a previous study in trauma, where mtDNA also induced NET production through TLR9 (32). mtDNA, but not nuclear DNA, robustly produces NETs in vitro. Pre-incubation of mtDNA with DNasel (and subsequent DNasel inactivation) completely blocks NET formation, as does the absence of TLR9. In chimeric lung transplants, the absence of TLR9 in either the lung donor or recipient decreases NET formation and lung injury in experimental PGD.

We have studied the effects of targeting NETs to reduce lung injury using two independent approaches—the use of PAD4 deficiency and treatment with DNasel. PAD4 is a critical enzyme that citrullinates histones to decondense chromatin during the process of NET formation. PAD4 deficient lung transplants have reduced citrullinated NETs and improved graft function. We have previously shown that intrabronchial DNasel treatment at the time of syngeneic lung transplantation reduces NETs and lung injury, and improves graft function (8). From our current study, it is now clear that DNasel had a dual mode of protection in PGD. DNasel efficiently degrades mtDNA, which is a major trigger of NETs in PGD, as well as efficiently degrading NET chromatin to limit tissue injury.

The treatment of ARDS is challenging, since the inflammatory/injury cascade is usually well-established by the time ARDS is clinically recognized. However, with PGD, the atrisk period is known and injury can be anticipated and preventive strategies implemented, such as DNasel delivered at the time of surgery or during ex vivo lung perfusion where NETs have been associated with worse recipient outcomes (33). These factors and the dual-action of DNasel in targeting NETs make it an appealing therapeutic option in PGD. Delivery of DNasel at the time of transplantation may prevent mtDNA-induced NET formation entirely, which could be important if NET-cleavage fragments promote immune responses in the setting of alloimmunity (34).

Our human studies have enhanced our understanding of the relevance of these pathways in PGD. BAL and plasma samples were obtained during the early PGD atrisk period and we confirmed previous results that elevation in BAL NETs, but not plasma, is associated with severe PGD status (8). Additionally, we discovered that mtDNA is also elevated in severe PGD, similar to results obtained in our experimental PGD model. We hypothesized that DNasel levels are reduced in severe PGD through consumption or underproduction. While absolute DNasel levels were not different in the PGD groups, we found that the NET/DNasel ratio was most associated with severe PGD. In other words, NET overproduction and/or relative DNasel deficiency correlates

Page 16 of 50

best with PGD. There are a variety of DNaseI polymorphisms that have been linked to reduced DNaseI levels, which should be studied in PGD susceptibility.

Our study is not without limitations. We did not determine the cellular origin of mtDNA in PGD, although we suspect that lung epithelial and endothelial cells are prominent sources since these cells are the target of ischemia-reperfusion damage. These cells likely contribute the majority of mtDNA in experimental PGD (35). However, we previously identified that platelets prominently accumulate in the PGD model (8), and platelets can release intact mitochondria and mitochondria-containing microparticles (36). Therefore, it is possible that platelets significantly contribute mtDNA in PGD as well. Although we found that mtDNA was sufficient to induce PGD, there are likely other DAMPs released during lung ischemia-reperfusion injury that could trigger NET formation that we did not study (12). For example, formylated peptides released from damaged mitochondria could be involved (35), although these peptides (e.g., fMLP) do not induce NET formation (37-39). Finally, in our chimeric lung transplant experiments, we did not identify the critical cell(s) that mediate mtDNA-TLR9 signaling. Neutrophils are likely involved based on our in vitro experiments and the protection observed in transplant combinations that included a deficiency of TLR9 in the transplant recipient (hematopoietic cells). However, our data also clearly indicate that donor lung cells are responding to TLR9 agonists to facilitate lung inflammation and injury in PGD. TLR9 is expressed on a variety of lung stromal cells and alveolar macrophages (40), which could mediate these responses.

In summary, our work presents broad evidence that mtDNA is generated during ischemia-reperfusion injury after lung transplantation and is a potent trigger of

pathogenic NET production via TLR9. It is likely that this pathophysiologic process is also important in other organ injuries and disease states that have reported dependency on the mtDNA-TLR9 axis. We also found a promising approach to treating NETinduced lung injury to improve lung transplant outcomes by targeting not only NETassociated chromatin, but also mtDNA that is an important upstream driver of NET formation.

Acknowledgment: The authors thank the Biological Imaging Development Center at the University of California, San Francisco for assistance with the intravital imaging experiments.

References

- Valapour M, Lehr CJ, Skeans MA, Smith JM, Carrico R, Uccellini K, Lehman R, Robinson A, Israni AK, Snyder JJ, Kasiske BL. OPTN/SRTR 2016 Annual Data Report: Lung. *Am J Transplant* 2018; 18 Suppl 1: 363-433.
- Suzuki Y, Cantu E, Christie JD. Primary graft dysfunction. Semin Respir Crit Care Med 2013; 34: 305-319.
- 3. Chambers DC, Cherikh WS, Goldfarb SB, Hayes D, Jr., Kucheryavaya AY, Toll AE, Khush KK, Levvey BJ, Meiser B, Rossano JW, Stehlik J. The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: Thirty-fifth adult lung and heart-lung transplant report-2018; Focus theme: Multiorgan Transplantation. *J Heart Lung Transplant* 2018; 37: 1169-1183.
- Christie JD, Kotloff RM, Ahya VN, Tino G, Pochettino A, Gaughan C, DeMissie E, Kimmel SE. The effect of primary graft dysfunction on survival after lung transplantation. *Am J Respir Crit Care Med* 2005; 171: 1312-1316.
- 5. de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. *Am J Respir Crit Care Med* 2003; 167: 490-511.
- Christie JD, Carby M, Bag R, Corris P, Hertz M, Weill D, Dysfunction IWGoPLG. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction part II: definition. A consensus statement of the International Society for Heart and Lung Transplantation. *J Heart Lung Transplant* 2005; 24: 1454-1459.

- Ross SD, Tribble CG, Gaughen JR, Jr., Shockey KS, Parrino PE, Kron IL. Reduced neutrophil infiltration protects against lung reperfusion injury after transplantation. *Ann Thorac Surg* 1999; 67: 1428-1433.
- Sayah DM, Mallavia B, Liu F, Ortiz-Munoz G, Caudrillier A, DerHovanessian A, Ross DJ, Lynch JP, 3rd, Saggar R, Ardehali A, Lung Transplant Outcomes Group I, Ware LB, Christie JD, Belperio JA, Looney MR. Neutrophil extracellular traps are pathogenic in primary graft dysfunction after lung transplantation. *Am J Respir Crit Care Med* 2015; 191: 455-463.
- Caudrillier A, Kessenbrock K, Gilliss BM, Nguyen JX, Marques MB, Monestier M, Toy P, Werb Z, Looney MR. Platelets induce neutrophil extracellular traps in transfusionrelated acute lung injury. *J Clin Invest* 2012; 122: 2661-2671.
- 10. Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med* 2017; 23: 279-287.
- Lefrancais E, Mallavia B, Zhuo H, Calfee CS, Looney MR. Maladaptive role of neutrophil extracellular traps in pathogen-induced lung injury. *JCI Insight* 2018; 3(3):e98178.
- 12. Todd JL, Palmer SM. Danger signals in regulating the immune response to solid organ transplantation. *J Clin Invest* 2017; 127: 2464-2472.
- Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, Hauser CJ. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* 2010; 464: 104-107.

- Headley MB, Bins A, Nip A, Roberts EW, Looney MR, Gerard A, Krummel MF.
 Visualization of immediate immune responses to pioneer metastatic cells in the lung.
 Nature 2016; 531: 513-517.
- 15. Looney MR, Thornton EE, Sen D, Lamm WJ, Glenny RW, Krummel MF. Stabilized imaging of immune surveillance in the mouse lung. *Nat Methods* 2011; 8: 91-96.
- Huang Z, Fasco MJ, Kaminsky LS. Optimization of Dnase I removal of contaminating DNA from RNA for use in quantitative RNA-PCR. *Biotechniques* 1996; 20: 1012-1014, 1016, 1018-1020.
- 17. Snell GI, Yusen RD, Weill D, Strueber M, Garrity E, Reed A, Pelaez A, Whelan TP, Perch M, Bag R, Budev M, Corris PA, Crespo MM, Witt C, Cantu E, Christie JD. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction, part I: Definition and grading-A 2016 Consensus Group statement of the International Society for Heart and Lung Transplantation. *J Heart Lung Transplant* 2017; 36: 1097-1103.
- 18. Lefrancais E, Ortiz-Munoz G, Caudrillier A, Mallavia B, Liu F, Sayah DM, Thornton EE, Headley MB, David T, Coughlin SR, Krummel MF, Leavitt AD, Passegue E, Looney MR. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature* 2017; 544: 105-109.
- 19. Hemmers S, Teijaro JR, Arandjelovic S, Mowen KA. PAD4-mediated neutrophil extracellular trap formation is not required for immunity against influenza infection. *PloS One* 2011; 6: e22043.

- 20. Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med* 2010; 207: 1853-1862.
- 21. Lewis HD, Liddle J, Coote JE, Atkinson SJ, Barker MD, Bax BD, Bicker KL, Bingham RP, Campbell M, Chen YH, Chung CW, Craggs PD, Davis RP, Eberhard D, Joberty G, Lind KE, Locke K, Maller C, Martinod K, Patten C, Polyakova O, Rise CE, Rudiger M, Sheppard RJ, Slade DJ, Thomas P, Thorpe J, Yao G, Drewes G, Wagner DD, Thompson PR, Prinjha RK, Wilson DM. Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. *Nat Chem Biol* 2015; 11: 189-191.
- 22. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol* 2012; 298: 229-317.
- 23. Saffarzadeh M, Juenemann C, Queisser MA, Lochnit G, Barreto G, Galuska SP, Lohmeyer J, Preissner KT. Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. *PloS One* 2012; 7: e32366.
- Puskarich MA, Shapiro NI, Trzeciak S, Kline JA, Jones AE. Plasma levels of mitochondrial DNA in patients presenting to the emergency department with sepsis. *Shock* 2012; 38: 337-340.
- 25. Zhang Q, Itagaki K, Hauser CJ. Mitochondrial DNA is released by shock and activates neutrophils via p38 map kinase. *Shock* 2010; 34: 55-59.
- 26. Nakahira K, Kyung SY, Rogers AJ, Gazourian L, Youn S, Massaro AF, Quintana C, Osorio JC, Wang Z, Zhao Y, Lawler LA, Christie JD, Meyer NJ, Mc Causland FR,

Waikar SS, Waxman AB, Chung RT, Bueno R, Rosas IO, Fredenburgh LE, Baron RM, Christiani DC, Hunninghake GM, Choi AM. Circulating mitochondrial DNA in patients in the ICU as a marker of mortality: derivation and validation. *PLoS Med* 2013; 10: e1001577.

- Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. A Toll-like receptor recognizes bacterial DNA.
 Nature 2000; 408: 740-745.
- Gu X, Wu G, Yao Y, Zeng J, Shi D, Lv T, Luo L, Song Y. Intratracheal administration of mitochondrial DNA directly provokes lung inflammation through the TLR9-p38 MAPK pathway. *Free Radic Biol Med* 2015; 83: 149-158.
- 29. Zhang JZ, Liu Z, Liu J, Ren JX, Sun TS. Mitochondrial DNA induces inflammation and increases TLR9/NF-kappaB expression in lung tissue. *Int j Mol Med* 2014; 33: 817-824.
- Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, Bolland S. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Science* 2006; 312: 1669-1672.
- 31. Hotz MJ, Qing D, Shashaty MGS, Zhang P, Faust H, Sondheimer N, Rivella S, Worthen GS, Mangalmurti NS. Red Blood Cells Homeostatically Bind Mitochondrial DNA through TLR9 to Maintain Quiescence and to Prevent Lung Injury. *Am J Respir Crit Care Med* 2018; 197: 470-480.

- 32. Itagaki K, Kaczmarek E, Lee YT, Tang IT, Isal B, Adibnia Y, Sandler N, Grimm MJ, Segal BH, Otterbein LE, Hauser CJ. Mitochondrial DNA Released by Trauma Induces Neutrophil Extracellular Traps. *PloS One* 2015; 10: e0120549.
- 33. Caldarone L, Mariscal A, Sage A, Khan M, Juvet S, Martinu T, Zamel R, Cypel M, Liu M, Palaniyar N, Keshavjee S. Neutrophil extracellular traps in ex vivo lung perfusion perfusate predict the clinical outcome of lung transplant recipients. *Eur Respir J* 2019; 53:e1801736.
- 34. Scozzi D, Wang X, Liao F, Liu Z, Zhu J, Pugh K, Ibrahim M, Hsiao HM, Miller MJ, Yizhan G, Mohanakumar T, Krupnick AS, Kreisel D, Gelman AE. Neutrophil extracellular trap fragments stimulate innate immune responses that prevent lung transplant tolerance. *Am J Transplant* 2019; 19: 1011-1023.
- 35. Scozzi D, Ibrahim M, Liao F, Lin X, Hsiao HM, Hachem R, Tague LK, Ricci A, Kulkarni HS, Huang HJ, Sugimoto S, Krupnick AS, Kreisel D, Gelman AE. Mitochondrial damage-associated molecular patterns released by lung transplants are associated with primary graft dysfunction. *Am J Transplant* 2019; 19: 1464-1477.
- 36. Boudreau LH, Duchez AC, Cloutier N, Soulet D, Martin N, Bollinger J, Pare A, Rousseau M, Naika GS, Levesque T, Laflamme C, Marcoux G, Lambeau G, Farndale RW, Pouliot M, Hamzeh-Cognasse H, Cognasse F, Garraud O, Nigrovic PA, Guderley H, Lacroix S, Thibault L, Semple JW, Gelb MH, Boilard E. Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. *Blood* 2014; 124: 2173-2183.

- 37. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol* 2018; 18: 134-147.
- 38. Yuen J, Pluthero FG, Douda DN, Riedl M, Cherry A, Ulanova M, Kahr WH, Palaniyar N, Licht C. NETosing Neutrophils Activate Complement Both on Their Own NETs and Bacteria via Alternative and Non-alternative Pathways. *Front Immunol* 2016; 7: 137.
- Remijsen Q, Kuijpers TW, Wirawan E, Lippens S, Vandenabeele P, Vanden Berghe T. Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death Differ* 2011; 18: 581-588.
- 40. Schneberger D, Caldwell S, Kanthan R, Singh B. Expression of Toll-like receptor 9 in mouse and human lungs. *J Anat* 2013; 222: 495-503.

Figure Legends

Figure 1. mtDNA is increased in experimental PGD and is completely degraded by **DNasel treatment.** (A-B) Lung intravital microscopy in OLT with donor lung minimal warm ischemia (A) or donor lung prolonged cold ischemia (PCI; B) (*mTmG* donor and recipient). Image is obtained within the first hour of reperfusion. SYTOX Green was administered (i.t. and i.v.) to highlight extracellular DNA (green). White circles indicate foci of intra-alveolar extracellular DNA. Scale bar = 20 µm. (C-D) Cytochrome C Oxidase 1 (specific for mitochondrial DNA) was amplified by gPCR from mouse BAL samples. All samples were obtained from WT mice in the following groups: nontransplanted mice (BAL only), lung transplants with no cold ischemia (immediate transplant), transplanted mice with donor lung PCI (18h), transplanted mice with PCI treated with either diluent or DNasel (2000 units) intra-bronchially at the time of transplantation. (C) Experimental PGD increased BAL mtDNA levels compared to controls. (D) mtDNA was undetectable in the PCI + DNasel model. Diluent was added in the group without DNasel. Data were analyzed using 1-way ANOVA (**B**) or Student's *t*-test (**C**) (n = 5-12). * $P \le 0.05$, *** $P \le 0.001$, **** $P \le 0.0001$.

Figure 2. mtDNA is sufficient to induce NET formation and lung injury after lung transplantation. (A) mtDNA measured by qPCR in mouse BAL samples. Donor lungs with minimal warm ischemia were instilled intrabronchially with mtDNA (50μ g in 15 µL volume) or diluent (15μ I) immediately prior to bronchial anastomosis. (B) Citrullinated NETs measured by citH3-DNA ELISA were higher in BAL samples of mice instilled with mtDNA compared to diluent controls. The mtDNA group had increased BAL protein (C), total white blood cells (WBC) (D) and neutrophils (E), and significantly reduced

Page 26 of 50

oxygenation capacity (**F**) versus diluent controls. Data were analyzed using Student's *t*-test (n = 7). * $P \le 0.05$, ** $P \le 0.01$, *** $P \le 0.001$.

Figure 3. mtDNA stimulates TLR9-dependent NET formation. Bone marrow neutrophils from WT and *TLR9^{-/-}* mice were isolated and stimulated with ionomycin (4 µM), mtDNA (100 µg/mL), DNasel-degraded mtDNA (100 µg/mL) or nuclear DNA (nucDNA, 100 µg/mL) for 4 hours at 37°C. NETs were measured from the cell-free supernatants by ELISA. (**A** and **B**) lonomycin stimulation increased NET production in WT and *TLR9^{-/-}* neutrophils but mtDNA only induced NETs in WT neutrophils. mtDNA digestion with DNasel completely eliminated NET induction in WT neutrophils. Nuclear DNA did not stimulate NET production in either WT or *TLR9^{-/-}* neutrophils. Data were analyzed using 1-way ANOVA (n = 8-9). ** $P \le 0.01$, *** $P \le 0.001$, **** $P \le 0.0001$. (**C**) Immunofluorescence staining of neutrophils from groups in **A** and **B** with antibodies against citrullinated histone 3 (blue) and neutrophil elastase (red), and DNA staining with SYTOX Green. The TLR9-specific agonist, ODN-1668 (10 µM), was added for this experiment. mtDNA and ODN-1668 stimulated NET formation in WT but not *TLR9^{-/-}* neutrophils. Scale bar = 50 µm.

Figure 4. TLR9 deficiency decreases NET content and lung injury in experimental PGD. (A) In experimental PGD using PCI of the donor lung, NETs are present in BAL fluid of WT transplants and reduced in any combination of lung transplant that includes $TLR9^{-/-}$ donor or recipients. (B-E) The lack of TLR9 in either lung donor or recipient improves lung injury as measured by BAL total protein (B), BAL WBCs (C), BAL neutrophils (D), or arterial blood pO₂ (E). Data were analyzed using 1-way ANOVA (*n* = 6-13). **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

Figure 5. PAD4 deficiency reduces citrullinated NET generation and improves

graft function. (**A**) Bone marrow neutrophils from WT or *PAD4^{-/-}* mice were stimulated with mtDNA (100 µg/mL) or DNaseI-degraded mtDNA (100 µg/mL) for 4 hours at 37°C. WT neutrophils stimulated with mtDNA produced citrullinated NETs, however no citH3-DNA complexes were produced in *PAD4^{-/-}* neutrophils stimulated with mtDNA. (**B**) In experimental PGD, BAL NETs (citH3-DNA complexes) were reduced in the setting of donor and recipient PAD4 deficiency. (**C-F**) The lack of PAD4 in both lung donor and recipient improves lung injury as measured by BAL WBCs (**C**), BAL neutrophils (**D**), or arterial blood pO₂ (**F**). (**E**) BAL total protein trended lower in the PAD4 deficient group, but was not significant (n.s.). Data were analyzed using 1-way ANOVA (**A**) or Student's *t*-test (**B-F**) (*n* = 7-12). ***P* ≤ 0.01, ****P* ≤ 0.001, *****P* ≤ 0.0001.

Figure 6. Severe PGD in humans is associated with increased BAL mtDNA and NETs and relative deficiency of BAL DNasel. (A-B) BAL and plasma mtDNA measurements (qPCR) in subjects with no PGD (PGD 0/1) or severe PGD (PGD 2/3). (C-D) BAL and plasma NETs (NE-DNA complexes) in no PGD or severe PGD. (E-F) BAL and plasma DNasel levels in no PGD or severe PGD. (G-H) BAL and plasma NET/DNasel levels in no PGD or severe PGD. Data were analyzed using the Mann-Whitney-Wilcoxon test. PGD 0/1 (n = 18); PGD 2/3 (n = 9). * $P \le 0.05$, ** $P \le 0.01$.











Figure 3











ONLINE DATA SUPPLEMENT

Mitochondrial DNA Stimulates TLR9-Dependent NET Formation in Primary Graft Dysfunction

Beñat Mallavia¹, Fengchun Liu¹, Emma Lefrançais¹, Simon J. Cleary¹, Nicholas Kwaan¹, Jennifer J. Tian¹, Melia Magnen¹, David M. Sayah⁴, Allison Soong¹, Joy Chen³, Rajan Saggar⁴, Michael Y. Shino⁴, David J. Ross⁴, Ariss Derhovanessian⁴, Joseph P. Lynch III⁴, Abbas Ardehali⁵, S. Sam Weigt⁴, John A. Belperio⁴, Steven R. Hays¹, Jeffrey A. Golden¹, Lorriana E. Leard¹, Rupal J. Shah¹, Mary Ellen Kleinhenz¹, Aida Venado¹, Jasleen Kukreja³, Jonathan P. Singer¹, Mark R. Looney^{1,2}

Supplemental Methods

Orthotopic lung transplant model of PGD

Orthotopic left lung transplants were carried out using the specified mouse genotypes, as previously described (1). The prolonged cold ischemia (PCI) model of PGD was induced by storing the left donor lung in sterile gauze soaked in Perfadex at 4°C for 18 hours prior to transplantation into the recipient mouse with minimal warm ischemia (1). Donor animals were anticoagulated with heparin (50 units injected into the inferior vena cava) followed by perfusion of the lung vasculature with 5 ml of ice-cold Perfadex solution (Xvivo Perfusion, Goteborg Sweden), clamping of the hilar structures, and removal of the left lung from the donor animal. A second group of lung transplants with no cold ischemia and minimal warm ischemia (immediate transplantation) were included as surgical controls for the PCI transplants. In selected experiments, DNasel (2,000 units in 15 µl diluent, Roche, Indianapolis, IN) vs. diluent alone (20 mM Tris-HCl, 1mM MgCl₂) was directly instilled into the donor lung bronchus prior to bronchial anastomosis. In selected experiments, mtDNA (50 µg in 15 µl diluent) vs. diluent alone (TE buffer from mitochondrial DNA extraction buffer: 15 µl) was directly instilled into the donor lung bronchus immediately prior to anastomosis. All animals were euthanized 8 hours after transplantation.

Lung intravital microscopy

A modified version (2) of the previously published method of stabilized lung imaging (3) was used. Immediately after minimal warm ischemia or PCI OLT (*mTmG* donor and recipient), mice were anesthetized with ketamine and xylazine and secured with tape to

a custom heated microscope stage. A small tracheal cannula was inserted, sutured into place, and attached to a MiniVent mouse ventilator (Harvard Apparatus). Mice were ventilated with a tidal volume of 10 µl/g (100% oxygen), a respiratory rate of 130–140 breaths per minute, and a positive-end expiratory pressure of 2-3 cm H2O. Isoflurane was continuously delivered to maintain anesthesia and mice were administered 300 µl of 0.9% saline solution i.p. every hour. To stain extracellular DNA, SYTOX Green was instilled intratracheally (50 µL of 1.0 mM solution) and intravenously (50 µL of 0.5 mM solution). Mice were placed in the right lateral decubitus position and a small surgical incision was made to expose the rib cage. A second incision was made into the intercostal space between ribs 4 and 5, through the parietal pleura, to expose the surface of the left lung. A flanged thoracic window with an 8 mm coverslip was inserted between the ribs and secured to the stage using a set of optical posts and a 90° angle post clamp (Thor Labs). 20-25 mm Hg of suction was applied to gently immobilize the lung. A XLUMPlanF 20X/0.95W water immersion objective (Olympus, Center Valley, PA) was then lowered into place over the thoracic window. Intravital imaging was performed using a Nikon A1R confocal microscope equipped with a 488 nm OBIS laser and a 561 nm Sapphire laser (Coherent, Palo Alto, CA). We captured a 0.4 mm² x-y surface every 1 µm over a 5 µm z-depth, capturing a complete 3D image every 10.5 seconds. Emission from SYTOX Green was detected with 500-550 nm filters and emission from tdTomato was detected with 570-620 nm filters (Semrock, Rochester, NY). Images were analyzed using Imaris 9.3 software (Bitplane, Zürich, Switzerland).

mtDNA and TLR9 quantitative PCR

mtDNA was quantified in cell-free BAL (1 μ L) fluid samples (mouse and human) by amplification of the mitochondria-specific Cytochrome C Oxidase 1 gene using mouse or human-specific primer/probe sets (Applied Biosystems, Foster City, CA). In preliminary experiments, mtDNA levels in mouse or human plasma were undetectable, and therefore plasma DNA was isolated using the Qiagen DNEasy kit (Germantown, MD) before proceeding to qPCR. TLR9 receptor expression was measured in bone marrow neutrophils from WT or *TLR9*^{-/-} mice using TLR9 primers and GAPDH primers (Taqman, Thermo Fisher Scientific, Waltham, MA).

In vitro NET generation and immunofluorescence

Bone marrow neutrophils were isolated using a 62% Percoll density gradient, and >90% neutrophil purity was assessed by cytospin preparations before all experiments. Neutrophils (5 x 10⁵) were plated and stimulated for 4 hours at 37°C and 5% CO₂ with 4 μ M ionomycin (Adipogen, San Diego, CA) or 100 μ g/mL of mtDNA or nuclear DNA extracted from mouse liver. mtDNA was isolated using the XIT Mitochondrial DNA kit (G-Biosciences, St. Louis, MO). Nuclear DNA was isolated with the NucleoSpin DNA RapidLyse kit (Macherey-Nagel, Düren, Germany). In select cases, mtDNA was pretreated with 70 U of DNasel (Roche) for 30 minutes at 37°C and then 5 minutes at 75°C to deactivate DNasel (5) prior to incubation with neutrophils. Cell-free supernatant was stored at -80°C for ELISA measurements. NET immunofluorescence was performed using 5 x 10⁴ neutrophils in 12-well chambers (Ibidi, Fitchburg, WI) at 37°C and 5% CO₂. After 4 hours of incubation with 4 μ M ionomycin, 100 μ g/mL mtDNA or 10

µM ODN-1668 (Invivogen, San Diego, CA), cells were fixed with 4% paraformaldehyde, incubated for 1 hour at room temperature with PBS/3% BSA, and stained at 4°C overnight with anti–neutrophil elastase G2 (sc-55549) and anti–citrullinated histone H3 (ab5103) antibodies. Secondary fluorescent antibodies were added for 1 hour at room temperature before staining with SYTOX Green for 10 minutes at room temperature (Invitrogen, Thermo Fisher Scientific). Images were captured using a Zeiss AXIO Imager M2 (Zeiss, Dublin, CA).

NET and DNasel ELISAs

NETs in cell culture supernatants and mouse or human BAL/plasma samples were quantified using ELISAs with mouse or human anti-neutrophil elastase primary antibodies (mouse: G-2 sc-55549; human: C17 sc-9520, both from Santa Cruz, Santa Cruz, CA) or anti-citrullinated histone-3 primary antibodies (mouse: ab5103, Abcam, Burlingame, CA; human: 17939, Cayman Chemical Company, Ann Arbor, MI) and an anti-DNA-HRP antibody (Cell Death Detection ELISA^{plus} Kit, Roche) as the detection antibody (4). TMB solution (DY999, R&D Systems, Minneapolis, MN) was used to develop the ELISAs. NE-DNA and citH3-DNA complexes are reported as optical density measurements (OD₄₅₀₋₄₉₀). DNasel levels in human BAL/plasma were measured using a DNasel ELISA (MBS2024612, MyBiosurce, San Diego, CA) following the manufacturer's instructions.

Acute lung injury measurements

A selective bronchoalveolar lavage (BAL) of the left lung was done by inserting a 20gauge catheter into the trachea, clamping the right hilum, and lavaging with 0.3 ml of cold PBS-EDTA (0.5M) flushed back and forth three times (1). To measure lung vascular permeability, the total protein concentration in the cell-free BAL was determined with a BCA protein assay kit (Thermo Scientific, Waltham, MA). Total white blood cells (WBC) were measured in the BAL samples using a Genesis cell counter (Oxford Science Inc, Oxford, CT), and cytospin preparations (Cytospin 3, Thermo Scientific) of 100uL of BAL with Hema-3 staining (Fisher Scientific, Hampton, NH) was used to determine the cellular differential.

Arterial blood gas measurements

At the end of the reperfusion period after OLT, mice were anesthetized, intubated, and placed on mechanical ventilation (10 μ l/g tidal volume, respiratory rate 120/min, PEEP 3 cmH2O) with 100% oxygen for 5 minutes. Blood was collected from the left ventricle into heparinized syringes for arterial blood gas measurements. Arterial blood gases were measured using a VetScan i-STAT 1 handheld analyzer and CG4+ cartridges (Abaxis, Union City, CA).

Human biospecimens

BAL and plasma samples were prospectively collected from two independent cohorts of subjects (UCSF and UCLA) on the morning of the first post-operative day following lung transplant surgery from April 2016 to August 2017. BAL was obtained as part of routine clinical bronchoscopy by the subjects' lung transplant clinician. All patients underwent bronchoscopy regardless of PGD status. BAL was collected in either the right middle lobe or lingula by instilling normal saline (UCSF: one 20 mL lavage; UCLA: two 60 mL lavages). PGD was defined and scored per international guidelines based on chest

radiography and oxygenation (6). PGD scores range from 0-3 with higher scores representing more severe disease. For the severe PGD group (PGD 2/3), BAL and plasma was available for 9 subjects who developed persistent severe PGD (grade 2 or 3 at 24, 48 and 72 hours post-transplant). We chose 20 BAL/plasma samples from subjects with PGD 0 or 1 (PGD 0/1) at all time points. In two PGD 0/1 subjects, BAL samples were unavailable (final n = 18), and in two other subjects, plasma was unavailable (final n = 18). The demographics and clinical characteristics of the subjects are shown in the Supplemental Table. Collected BAL and plasma were immediately processed and stored at -80°C. BAL WBC counts and the cellular differential were quantified as described with mouse BAL samples.

Statistics

All in vivo and in vitro experiments were repeated a minimum of three independent times. Unless otherwise noted, all mouse data are presented as mean \pm SEM and human subject data are presented as min-to-max whiskers and box plots showing the median and interquartile ranges. For the mouse experiments, to determine significance, tests were used as appropriate (2-tailed Student's *t*-test to compare 2 groups, 1-way ANOVA with Sidak's multiple comparisons tests to compare more than 2 groups where relevant). For the human studies, data were compared using the Mann-Whitney-Wilcoxon test. C-statistics from receiver operator characteristic curves were generated to determine which of the mtDNA, NETs, or NET/DNasel measurements early after transplantation were most predictive of subsequent PGD development. A *P* value less than or equal to 0.05 was considered significant. All statistical analyses were performed

using GraphPad Prism 7.0 (GraphPad Software, Inc. San Diego, CA) and STATA v15.1 (StataCorp, College Station, TX).

Supplemental Figure Legends

Supplemental Figure 1. TLR9 mRNA is present in WT neutrophils and absent in *TLR9-/-* cells. TLR9-specific mRNA was amplified by qPCR from bone marrow neutrophils isolated from WT and *TLR9-/-* mice using Taqman primers/probes. TLR9 expression was only observed in WT cells. Data were analyzed using Student's *t*-test (n = 4 per group). * $P \le 0.05$.

Supplemental Figure 2. BAL cell counts in humans after lung transplantation. (A-

C) In BAL samples obtained on the first post-transplantation day, there were no differences in total WBCs, neutrophils, or macrophages in human subjects with no PGD or severe PGD. We detected no or very few lymphocytes in either PGD group (data not shown). Data were analyzed using the Mann-Whitney-Wilcoxon test. PGD 0/1 (n = 18); PGD 2/3 (n = 9).

Supplemental Figure 3. Receiver operating curves for human BAL biomarkers after lung transplantation and PGD prediction. (A-D) ROC curves for BAL mtDNA, NETs, DNasel, and NETs/DNasel and ability to predict severe PGD. Area under the curve (AUC) analysis indicates that BAL NETs/DNasel (D) was the best predictor of severe PGD.

Supplemental Movie Legend

Supplemental Movies 1 and 2. Confocal lung intravital microscopy in OLT with minimal warm ischemia (Movie 1) and with PCI (Movie 2) during the first hour of reperfusion. In both cases mTmG donor and recipients were used and SYTOX Green (i.t. and i.v.) was administered to highlight extracellular DNA (green). Scale bar = 20 μ m.

Supplemental References

- Sayah DM, Mallavia B, Liu F, Ortiz-Munoz G, Caudrillier A, DerHovanessian A, Ross DJ, Lynch JP, 3rd, Saggar R, Ardehali A, Lung Transplant Outcomes Group I, Ware LB, Christie JD, Belperio JA, Looney MR. Neutrophil extracellular traps are pathogenic in primary graft dysfunction after lung transplantation. *Am J Respir Crit Care Med* 2015; 191: 455-463.
- Headley MB, Bins A, Nip A, Roberts EW, Looney MR, Gerard A, Krummel MF.
 Visualization of immediate immune responses to pioneer metastatic cells in the lung. *Nature* 2016; 531: 513-517.
- 3. Looney MR, Thornton EE, Sen D, Lamm WJ, Glenny RW, Krummel MF. Stabilized imaging of immune surveillance in the mouse lung. *Nat Methods* 2011; 8: 91-96.
- Lefrancais E, Mallavia B, Zhuo H, Calfee CS, Looney MR. Maladaptive role of neutrophil extracellular traps in pathogen-induced lung injury. *JCI Insight* 2018; 3(3):e98178.
- Huang Z, Fasco MJ, Kaminsky LS. Optimization of Dnase I removal of contaminating DNA from RNA for use in quantitative RNA-PCR. *Biotechniques* 1996; 20: 1012-1014, 1016, 1018-1020.
- Snell GI, Yusen RD, Weill D, Strueber M, Garrity E, Reed A, Pelaez A, Whelan TP, Perch M, Bag R, Budev M, Corris PA, Crespo MM, Witt C, Cantu E, Christie JD. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction, part I: Definition and grading-A 2016 Consensus Group statement of the International Society for Heart and Lung Transplantation. *J Heart Lung Transplant* 2017; 36: 1097-1103.

Supplemental Table. Demographics and clinical characteristics of PGD cohort.

	PGD 2/3	PGD 0/1	p-value
Ν	9	20	
Age, years	63 ± 8.3	58 ± 14.4	0.38
Female	5 (55%)	9 (45%)	0.70
Race/Ethnicity			
White, Non-Hispanic	4 (44%)	15 (75%)	0.58
Asian	0 (0%)	1 (5%)	
Black	0 (0%)	2 (10%)	
Hispanic	4 (44%)	2 (10%)	
Other	1 (11%)	0 (0%)	
Diagnostic Category*			
Group A (Obstructive lung disease)	0 (0%)	4 (20%)	0.55
Group B (Pulmonary hypertension)	1 (11%)	1 (5%)	
Group C (Suppurative lung disease)	1 (11%)	3 (15%)	
Group D (Pulmonary fibrosis)	7 (77%)	12 (60%)	
Lung Allocation Score	40.9 ± 8.8	49.2 ± 18.1	0.20
Body mass index, kg/m ²	28.3 ± 3.7	25.8 ± 4.2	0.14
Mean pulmonary artery pressure, mmHg	27.6 ± 12.1	24.2 ± 5.6	0.31
Ischemic time, minutes	290 ± 105	321 ± 74	0.37
Lowest PaO ₂ /FiO ₂ ratio on post-operative day 0	254 ± 60	392 ± 44	<.0001

Data presented as n (%) or mean ± SD.

*Diagnostic categories used for calculation of the Lung Allocation Score.



Supplemental Figure 1

Supplemental Figure 1



Supplemental Figure 2

Supplemental Figure 2



Supplemental Figure 3

Supplemental Figure 3



Still of Supplementary Video 1



Still of Supplementary Video 2