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Neutrophil extracellular trap fragments stimulate innate immune responses that prevent lung transplant tolerance

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Abstract

Neutrophil extracellular traps (NETs) have been shown to worsen acute pulmonary injury including after lung transplantation. The breakdown of NETs by DNAse-1 can help restore lung function, but whether there is an impact on allograft tolerance remains less clear. Using intravital 2-photon microscopy, we analyzed the effects of DNAse-1 on NETs in mouse orthotopic lung allografts damaged by ischemia-reperfusion injury. Although DNAse-1 treatment rapidly degrades intragraft NETs the consequential release of NET fragments induces prolonged interactions between infiltrating $CD4^+$ T cells and donor-derived antigen presenting cells. DNAse-1 generated NET fragments also promote human alveolar macrophage inflammatory cytokine production and prime dendritic cells for alloantigen-specific $CD4^+$ T cell proliferation through activating Toll-like receptor (TLR) - Myeloid Differentiation Primary Response 88 (MyD88) signaling pathways. Furthermore, and in contrast to allograft recipients with a deficiency in NET generation due to a neutrophil-specific ablation of Protein Arginine Deiminase 4 (PAD4), DNAse-1 administration to wildtype recipients promotes the recognition of allo- and self-antigens and prevents immunosuppression-mediated lung allograft acceptance through a MyD88-dependent pathway. Taken together, these data show that the rapid catalytic release of NET fragments promotes innate immune responses that prevent lung transplant tolerance.

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Disclosure

The authors of this manuscript have no conflicts of interest to declare as described by the *American Journal of Transplantation*.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

Introduction

NETosis is a potent neutrophil effector function that immobilizes and kills extra-cellular pathogens through the release of decondensed chromatin decorated with antimicrobial proteins including myeloperoxidase (MPO), citrullinated histones, elastase and metalloproteases[1]. Since many of the antimicrobial contents of NETs have intrinsic cytotoxic activity, they also have the potential to cause bystander injury to host cells[2]. In the lung, this has been suspected during infectious exacerbations of Cystic Fibrosis (CF), Chronic Pulmonary Obstructive Disease, Bacterial Pneumonia, and Tuberculosis[3–6]. However, more recent work has demonstrated that NETs also form in response to non-infectious tissue injury. NETs have been reported to promote inflammation in models of primary lung graft dysfunction (PGD)[7], transfusion-related acute lung injury[8] and ventilation-induced acute lung injury[9, 10].

The depletion of NETs from injured lungs has been shown to inhibit pulmonary edema, promote microcirculation, and attenuate fibrosis[7–9, 11]. NET chromatin is particularly sensitive to DNAse-1 (DNAse) mediated digestion[12]. These observations have supported the use of recombinant DNAse as a therapy for several NET-associated lung diseases. DNAse is currently an FDA approved treatment for CF patients where it used to improve lung function by reducing the viscosity of chromatin-containing sputum[13]. DNAse has also shown promise to treat severe Respiratory Syncytial Virus-induced Bronchiolitis and acute asthmatic exacerbations both of which have been linked to NETosis[14, 15]. However, DNAse treatment has also been reported to have unintended consequences. DNAse administration in some unresponsive CF patients appears to release lung-damaging protease activity associated with NETosis[16]. Additionally, DNAse treatment of CF sputum has been reported to induce lung hemorrhage in mice by increasing the elastase activity[17]. Finally, in a mouse model of Gout-induced NETosis DNAse treatment promoted neutrophilia and expression of CXCL1, an ELR^+-CXC analog of the neutrophilic cytokine IL-8[18].

Recent work by Sayah and colleagues has demonstrated that clinical lung transplant recipients with PGD have excessive NETosis[7]. They also show that the administration of DNAse to mice with ischemically-injured syngeneic lung grafts improves pulmonary function. However, how DNAse regulates immunity to lung allografts remains unknown. Here we investigated the effects of disrupting NETs in mice that received fully-MHC mismatched lung transplants. Despite an early improvement in transplant function, DNAse generates NET fragments that prevent lung allograft tolerance through the activation of TLR-dependent innate immune responses controlled by the TLR signal transducing molecule MyD88.

Methods

Human Studies

Under the guidelines of the Washington University School of Medicine Institutional Review Board (IRB #201012829) written consent was obtained from patients and healthy volunteers after explanation of the nature and possible consequences of participating in this study.

Mice and Lung Transplants

All mice experiments were conducted with an approved IACUC Washington University protocol. C57BL/6J (B6), CD11c-Cre-EGFP (CD11c EGFP), CD45.1^{+} C57BL/6, Balb/c, MyD88$^{-/-}$, Trif$^{-/-}$, PAD4$^{fl/fl}$, Mrp8Cre and Caspase-1$^{-/-}$ mice were obtained from Jackson Labs. Mrp8Cre mice were crossed with PAD$^{fl/fl}$ mice generate to PAD4$^{\Delta/\Delta}$ mice. Balb/c.MyD88$^{-/-}$ mice and littermate controls were obtained from Oriental Bioservices in Japan. LysM-eGFP (B6) mice were obtained from Klaus Ley (La Jolla Institute for Allergy and Immunology). All mice except for Balb/c-derived mice are on a B6 background. Six to 12-week donor and eight to ten week recipient mice were used in all experiments. Orthotopic lung transplants were performed as previously described by our group[19] with the following exceptions to induce injury. After procurement donor lungs were stored in 4 ^{O}C LPDG preservation for 18 hours and then transferred to 25 ^{O}C LPDG solution for one hour prior to orthotopic engraftment to induce ischemia reperfusion injury. Minimal ischemia time was accomplished with 1 hour of 4 ^{O}C LPDG preservation prior to engraftment. Lung allograft recipients for tolerance studies were immunosuppressed with cyclosporine A (10mg/kg body weight /day; Sigma) and methylprednisolone (1.6 mg/kg body weight/day; Sigma) as previously described by our group[20]. Arterial blood gases were measured using an iSTAT Portable Clinical Analyzer (iSTAT) at a FiO_2 of 1.0 after the right pulmonary hilum was clamped for 5 minutes. For histology, the left lung grafts were harvested, inflation fixed in formaldehyde, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and evaluated for inflammation by a blinded pathologist. All mice received care in compliance with the “Guide for the care and use of laboratory animals” prepared by the National Academy of Sciences and published by the National Institutes of Health and the “Principles of laboratory animal care”.

2P intravital imaging studies

The left lung allograft was exposed by removal of chest wall between the 3rd and 7th ribs. A cover glass slide was adhered to lung allograft using tissue glue (VetBond) applying minimal pressure so as not to disturb flood flow. Intragraft neutrophils, vessels and NETs were respectively probed with PE-Ly6G Ab (2 μg, clone 1A8;E-bioscience), 655-nm Q-dots (20 μl; Invitrogen) and 1:1000 dilution of Sytox Green (10 μl; Invitrogen) administered intravenously 30 minutes after engraftment. Fields were excited with 890 nm laser and images were collected through 480 nm and 560 nm filters to image neutrophils in green and NET structures in blue. To image NET dissolution recombinant DNase-1 (10 μg/g body weight; Sigma) and Q-dots (10 μl) were co-administered i.v. 30 minutes after the start of imaging. To analyze the effects of DNAse treatment under conditions where NET formation is inhibited, 0.3 mg of Cl-Amidine (Sigma) was administered in 200 μl of saline i.p. to recipients one hour prior to engraftment. Sequential z-sections (24, 2.5 μm each) were acquired in an imaging volume of 200 × 225 × 60 μm^3. Analyses were performed with Imaris (Bitplane, Zurich, Switzerland). Associations between CD4^{+} T cells and CD11c^{+} cells were defined as physical interactions that lasted greater than 15 seconds. To image these interactions, we used 920 nm excitation and emission filters of 495 and 560 nm. For each lung transplant, at least 5 areas were examined up to approximately 50 μm deep. Data shown has been pooled from at least 4 mice per group.

NET ELISA

NETs release in culture and plasma was quantified with Quanti-IT Pico Green double stranded (ds) DNA kits (Thermo Fisher Scientific) using lambda DNA as quantitation standards in accordance with manufacturers recommendations. To capture NET fragments from BALF 100 μg of rabbit polyclonal Chip grade Abs specific for citrullinated histone H3 (Abcam) or 500 μg control rabbit polyclonal Abs (Jackson Labs) were conjugated to 1.7 mg of 0.3 mm diameter IDC UltraClean Amidine Latex beads (Fisher Thermo Scientific) in accordance with manufacturers recommendations. BALF was split into two 1.5 ml aliquots and incubated with either 250 μg H3 citrullinated Ab-beads or control Ab-beads at 4 °C for 3 hours. Beads were then washed twice in cold PBS and directly quantified for dsDNA content with Pico Green relative to dsDNA content adsorbed to control Ab-beads. MPO-DNA complex detection in circulating plasma was conducted with MPO ELISA kit plates (ThermoFisher Scientific) precoated with MPO capture Abs. Then following plasma incubation for 3 hours at 4° C and 3 washes of cold ELISA washer buffer plates were incubated with anti-DNA peroxidase conjugated Abs from a Cell Death Detection ELISA kit (Sigma), washed three times, and incubated with ABTS solution (Sigma) for 20 minutes. Complexes were quantified as optical density at 405 nm using a Synergy Multimode reader (Biotek).

NET fragment preparation

Human neutrophils were isolated from healthy human volunteers using an Easy Sep neutrophil negative selection kit (Stemcell). NETs were generated from 4×10^6 neutrophils following stimulation with 10 μg/ml PMA for 4 hours in complete medium with 10% human AB serum in a 150 cm^2 culture dish. Isolation of NETs from neutrophils were conducted in accordance with methods previously reported[21]. Briefly, culture medium was carefully removed, and replaced with cold HBSS and NETs were released from neutrophils through gentle pipetting and clarified from cell debris by centrifugation at 150 × g for 20 minutes leaving the supernatant enriched with NETs. NETs were then pelleted at 20,000 × g for 30 minutes in 4 °C re-suspended in 0.5 ml of HBSS. For fragmentation NETs were incubated with 2.5 units/ml DNAse-1 with 0.5 mM $MgCl_2$, equivalent to the amount of free Mg^{2+} in human blood[22], were monitored for fragmentation by gel electrophoresis via ethidium bromide staining. NET fragments (NET^{frag}) used for experiments were removed from the reaction at 3 to 4 hours after exposure and ranged from 0.2 to 25 kilodaltons. Completely digested NETs ($NETs^{ext}$) were generated by 18 hours of exposure for DNAse. $NETs^{Frag}$ and NET^{Ext} and supernatents from PMA-stimulated NETs (NET Sup) were stored at - 70 °C prior for use in experiments. For experiments with monocyte-derived human dendritic cells NETs were also sheared (NET^{sonic}) with Qsonica sonicator for 3 discrete 10 sec pulses at 40% amplitude while on ice.

Primary human cell culture.

Human alveolar macrophages were isolated from transplant BAL of donor lungs approximately 2 hours after reperfusion by plastic adherence in DMEM (Gibco) complete medium supplemented with 20% human AB serum (Corning) and 20 ng/ml human M-CSF-1 (Peprotech) for 48 hours before the addition of NET fragments. Following

stimulation with NET preparations for 24 hours supernatant cytokine accumulation was measured for indicated cytokines with ProCarta Luminex assays (ThermoFisher) in accordance with manufacturer's recommendations. To generate human monocyte-derived dendritic cells, peripheral blood monocytes were isolated with the Easy Sep monocyte enrichment kit (Stemcell) from healthy human volunteers. 5×10^6 monocytes per ml were added in 6-well plates and cultured in DMEM complete medium supplemented with 10% AB serum, human GM-CSF (100ng/ml; Peprotech) and IL-4 (50 ng/ml; Peprotech) for 6 days with refreshment of media every 2 days prior to use in experiments.

TLR reporter lines, dendritic cell maturation and T cell adoptive transfer

Human TLR2, 3, 4 and 9 reporter HEK-Blue (Invivogen) cell lines were grown in accordance with manufacturer's recommendations and following overnight stimulation with indicated NET preparations were assessed for TLR activity using QuantiBlue Assays (Invivogen) at 605 nm. Bone marrow from indicated mice was flushed from femurs and tibias, depleted of red blood cells with ACK lysing buffer (Lonza) and depleted of T lymphocytes using CD90.2 Ab conjugated immunomagnetic beads (Miltenyi). The remaining cells were then cultured for 7 days in IMDM complete media (Gibco) supplemented with 10% FCS (Biowhittaker), mouse recombinant GM-CSF (30 ng/ml; Peprotech) and IL-4 (10ng/ml; Peprotech). For T cell and dendritic cell analysis, all Abs were from ebiosciences. Mouse dendritic cell maturation was analyzed with IA^b (AF6–120.1), CD80 (clone 16–10A1), CD86 (clone GL1) and CD40 (HM40–3) Abs. Human monocyte derived dendritic cell maturation was assessed with HLA-DR (clone LN3), CD80 (clone 2D10.4) and CD83 (clone HB15e). For proliferation assays or adoptive transfer experiments naïve $CD4^+$ T cells were isolated from FACS sorts of B6 mice disrupted spleens and lymph nodes with the following Abs :anti-NK-1.1-FITC (clone PK136), anti-CD45R-FITC (clone RA3–6B2), anti-CD11c-FITC (clone N418), anti-CD11b-FITC (clone M1/70), anti-CD69-FITC (clone H1.2F3), anti-CD4-PerCP-Cy 5.5 (clone RM4–5), anti-CD90.2-APC (clone 53–2.1), anti-CD62L-APC-Alexa 780 (clone MEL-14), anti-CD44-FITC (clone IM7), anti-Gr1-FITC (clone RB6–8C5) and anti-CD25-FITC (clone PCS1) through a $CD90.2^+$ $CD4^+$ $CD62L^+$ $CD25^-$ $CD69^-$ $CD44^-$ CD11c- CD11b- NK1.1-CD45R-Gr1- gate using a Synergy 3200 BSC machine (Sony Biotechnology). Naïve $CD4^+$ T cells were stained with 5 mM CFSE and 3×10^5 of these cells were co-incubated with indicated stimulated 10^5 dendritic cells in roundbottom 96 well plates for 72 hours.

Intragraft T cell accumulation and antigen specificity

Lungs allografts were digested with collagenase and dispase as previously described[23]. Cell suspension was surface stained with CD45.2 (Biolegend, clone 104), CD8a (eBioscience, clone 53–6.7), CD4 (eBioscience, clone RM4–5), CD90.2 (eBioscience, clone 53–2.1). For intracellular IFN-γ and IL-17A staining cell suspensions were stimulated for 4 hours with 20 ng/ml PMA (Sigma), and 1 μg/ml of Ionomycin (Sigma) in the presence of 1 μg/ml Golgi Plug (BD Biosciences) for 4 hours. Cells were fixed and permeabilized with a BD Cytofix/Cytoperm kit (BD Biosceinces) and then stained with IFN-γ Ab (eBioscience, clone XMG1.2) and IL-17A Ab (Biolegend, clone TC11–18H10.1). For antigen specificity measurements, $CD4^+$ T cells were fractionated by positive selection using $CD4^+$ immunomagnetic beads (Miltenyi) from allograft cell suspensions and co-cultured in 3:1

ratio with irradiated T cell-depleted Balb/c or B6 cell splenocytes for 96 hours pulsed with 1 μg/ml k-alpha tubulin or collagen V (both were obtained from Dr. T. Mohannakumar, St. Joseph's Hospital, Phoenix AZ). IFN-γ and IL-17A/F were measured with uncoated ELISA kits from Invitrogen in accordance with manufacturer's recommendations.

Statistical Analysis

Significant differences were evaluated with the non-parametric Kruskal-Wallis (Dunn`s test for multiple comparison) or with the one-way ANOVA (Tukey`s test for multiple comparisons) if normally distributed. Normal distribution was calculated using Shapiro-Wilks test. A two-way ANOVA when appropriated were used to analyze data with more than two groups. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc.) and a P value < 0.05 was considered significant.

Results

DNAse treatment rapidly degrades allograft NETs.

To analyze the effects DNAse-1 (DNAse) on NETs in lung recipients we utilized a mouse model of orthotopic lung transplant-induced ischemia-reperfusion injury where major histocompatibility class I and II mismatched Balb/c (Balb) lungs were engrafted into C57BL/6 (B6) recipients. Approximately 30 minutes after reperfusion NETs were imaged by intravital 2-photon microscopy (Fig. 1 & Movies 1–3). In contrast to saline vehicle treatment, DNAse administration led to NET dissolution within five minutes after administration. Additionally, NETs were not detected in lung recipients treated in Cl-Amidine, which prevents NETosis through inhibiting proline arginase deaminase-mediated citrullination of histones[24].

DNAse treatment improves allograft function but releases NET fragments

We asked if DNAse-treatment improves pulmonary function of ischemia-reperfusion injured lung allografts (Fig. 2a). When compared to saline vehicle or Cl-Amidine-treatment, DNAse administration significantly improved oxygenation of injured allografts. Furthermore, lung function in allograft recipients treated with both Cl-Amidine and DNAse was comparable to recipients that only received Cl-Amidine indicating that DNAse-mediated improvement in lung function is dependent on NET digestion. Based on previous observations that DNAse can generate NET fragments[25] we measured levels of neutrophil chromatin in the peripheral blood of lung allograft recipients (Fig. 2b). We detected significantly elevated concentrations of MPO-DNA complexes in the plasma at 1.5 and 18 hours after DNAse treatment. In contrast, Cl-Amidine pretreatment of lung recipients inhibited DNAse-mediated elevation of circulating MPO-DNA complexes. Consistent with these observations was a similar pattern of circulating plasma DNA accumulation (Fig. 2c). We also captured NET fragments in the bronchoalveolar lavage (BAL) fluid of DNAse-treated recipients using beads conjugated to Abs specific for citrullinated histone 3 (cit-H3) (Fig. 2d). Following DNAse treatment cit-H3 Ab containing beads captured between 2.7 and 6.1 μg DNA per allograft recipient. In contrast, bronchioalveolar lavage fluid (BALF) from saline vehicle-treated allografts had approximately 5-fold more NETs indicating that DNAse activity was present in the airway. To examine the possibility that NET fragments within the airway was not due to suboptimal

DNAse administration we performed DNAse dose responses studies on B6 mice that had undergone left lung hilar clamping, a form of warm ischemia pulmonary injury that has been previously shown to generate airway NETs[7] (Fig. 2e). Increasing DNAse dose beyond 10μg/g bodyweight only had a modest effect reducing airway NET fragments suggesting that remaining airway NETs was not due to insufficient administration.

NET fragments stimulate inflammatory cytokine production in human APCs

Because NETs harbor molecules that can promote antigen presenting cell (APC) maturation[26] we assessed the effect of NET fragment stimulation on human alveolar macrophages and monocyte-derived dendritic cells. To this end, we generated NET fragments from PMA-stimulated human neutrophils (Fig. S1). NET fragments induced cytokine expression implicated in T_h1 and T_h17 development (IL-12p70, IL-1β, IL-6 and IL-23) and antigen processing (IFN-α) from alveolar macrophages (Fig. 3a & Fig. S2). Additionally, NET fragments produced either by DNAse or sonication promoted the upregulation MHC Class II and the costimulatory molecules CD80 and CD83 on human monocyte-derived dendritic cells (Fig. 3b). By contrast, equivalent amounts of NET chromatin that were completely digested by DNAse were significantly less capable of activating alveolar macrophages and dendritic cells. Altogether, these data demonstrate that NETs fragments drive inflammatory gene expression in human macrophages and dendritic cells.

NET fragments drive TLR-MyD88 dependent dendritic cell maturation

We next asked if NETs fragments stimulate inflammatory responses through TLRs. NET fragments promoted TLR2, −4 and −9 but not −3 activation in human TLR reporter cell lines (Fig. 4a). We further analyzed NET fragment-mediated TLR activation utilizing mouse bone marrow-derived dendritic cells (BMDC) deficient in Trif or MyD88, TLR-signaling transducing molecules that promote inflammatory responses, including maturation[27] (Fig. 4b). NET fragments sharply induced the upregulation of MHC Class II, CD40, CD80 and CD86 in wildtype B6 and $Trif^{-/-}$ BMDCs but not in $MyD88^{-/-}$ BMDCs. Notably, NET fragments also simulated the maturation of $Caspase1^{-/-}$ BMDCs[28], demonstrating that TLR-MyD88 but not IL-1R/18R-MyD88 signaling promotes dendritic cell maturation in response to NET fragments. This pattern of TLR-signaling pathway activation was additionally reflected in the ability of NET fragments to promote BMDC-mediated proliferative responses from naïve allogeneic $CD4^+$ T cells (Fig. 4c). While NET fragment-stimulated B6, $Trif^{-/-}$ and Caspase $1^{-/-}$ BMDCs potently drove $CD4^+$ T cell proliferation, $MyD88^{-/-}$ BMDC-mediated proliferative responses were only slightly enhanced over untreated cultures. Taken collectively, these data demonstrate that NET fragments promote dendritic cell maturation through TLR-MyD88 signaling pathways.

NET fragments stimulate prolonged interactions between donor-derived APCs and graft-infiltrating $CD4^+$ T cells

Previous work by our group has shown that $CD4^+$ T cell responses following lung transplantation is initiated within allograft tissue[29]. Prolonged associations between $CD4^+$ T cells and APCs is required for optimal antigen-specific adaptive immune responses[30]. We therefore measured interaction times between graft-infiltrating $CD4^+$ T cells and donor-

derived CD11c^{+} APCs using intravital 2-photon microscopy (Fig. 5a–b; Movies 4–6). When compared to lung recipients treated with saline vehicle we observed a significant increase in CD4^{+} T cell - CD11c^{+} APC interaction times in allografts that received NET fragments or DNAse. To further confirm these observations, we adoptively transferred naïve CD45.1^{+} CD44^{-} CD62L^{+} CD4^{+} T cells into lung allograft recipients that received DNAse or NET fragments and 48 hours later analyzed their activation within allograft tissue by flow cytometric analysis (Fig. 5c). DNAse and NET fragment administration promoted more naïve CD44^{-} CD62L^{+} CD4^{+} T cell differentiation into effector CD44^{+} CD62L^{+} and CD44^{+} CD62L^{-} subsets than did recipients treated with saline vehicle.

NET fragments prevent lung transplant acceptance

To further explore the relevance of NET fragment-mediated priming of APCs we transplanted ischemically-injured Balb/c wild-type (Balb.WT) or Balb/c MyD88$^{-/-}$ deficient (Balb.MyD88$^{-/-}$) lungs into immunosuppressed B6 recipients that received either saline, NET fragments or DNAse. On post-operative day (POD) 7 lung recipients were assessed for histopathological evidence of intragraft inflammation (Fig. 6). Lung recipients that received saline, irrespective of donor MyD88 expression, accepted their allografts. MyD88$^{-/-}$ allografts were also accepted in recipients that received either NET fragments or DNAse. In contrast, wild-type allografts were acutely rejected in NET fragment or DNAse treated recipients. We considered the possibility that DNAse is preventing tolerance in a manner independent of NET digestion. Accordingly, we transplanted ischemically-injured Balb.WT lungs into immunosuppressed recipients that are deficient in NET generation due to PAD4 ablation specifically within their neutrophil compartment (Mrp8Cre × PAD4 $^{fl/fl}$; PAD4 $^{\Delta/\Delta}$)[31]. While the administration of NET fragments induced allograft rejection in PAD4 $^{\Delta/\Delta}$ recipients, DNAse treatment failed to prevent lung allograft acceptance in PAD4 $^{\Delta/\Delta}$ recipients. We next asked if DNAse-mediated prevention of lung allograft tolerance is dependent on ischemia-reperfusion injury-induced NETosis (Fig. S3a–b). To this end, we attenuated ischemia-reperfusion injury be reducing the cold preservation time of Donor Balb. WT lungs from 18 hours to 1 hour prior to transplantation into B6 recipients. Lung allografts that underwent 1 hour of cold storage time approximately seven-fold less intragraft NET generation relative to allografts that underwent 18 hours of cold preservation. Moreover, lung allografts that were cold preserved for one hour were tolerated by immunosuppressed B6 recipients despite DNAse treatment indicating that severe ischemia-reperfusion is responsible for the NET generation that promotes DNAse-mediated alloimmune responses. We also determined the effects of two injections of DNAse into immunosuppressed B6 recipients of ischemically injured Balb. WT allografts (Fig. S3c–d). Although we observed a modest reduction in airway NETs, allografts still rejected underscoring the difficulty of using DNAse to completely degrade NETs within allografts. Collectively, these data show that NET fragments generated by DNAse administration prevent immunosuppression-mediated lung allograft acceptance.

Because lung allograft rejection is coupled to adaptive immune responses against graft antigens we analyzed the abundance of effector T cells within transplanted lungs (Fig. 7a–b & Fig. S4). In immunosuppressed B6 recipients of Balb.WT lungs, DNAse or NET fragment administration significantly increased the abundance of allograft - infiltrating IFN- γ^{+} CD4^{+},

IL-17A$^+$ CD4$^+$ and IFN- γ^+ CD8$^+$ T cells. In accordance with their inability to generate NETs, DNAse treatment of PAD4$^{\Delta/\Delta}$ recipients of Balb.WT lungs failed to enhance intragraft effector T cell accumulation. Furthermore, Balb.MyD88$^{-/-}$ allografts did not accumulate effector T cells in response to DNAse or NET fragment administration.

We next assessed antigen specificity of intragraft CD4$^+$ T cells from recipients that received NET fragments through measuring antigen recall-mediated IFN- γ and IL-17A expression (Fig. 8a–b). Alloantigen-challenged intragraft CD4$^+$ T cells from wild-type allografts, irrespective of whether they were transplanted into B6 or PAD4 $^{\Delta/\Delta}$ recipients, produced higher amounts of IFN- γ and IL-17A when compared to CD4$^+$ T cells from MyD88$^{-/-}$ allografts. The accumulation of autoreactive CD4$^+$ T cells with specificity to the pulmonary proteins Collagen V (ColV) and K-α1 tubulin (Kα1T) has been linked to decreased lung transplant survival[32, 33]. Challenge of wild-type allograft-infiltrating CD4$^+$ T cells with ColV or Kα1T from recipients treated with NET fragments induced significantly higher IL-17A generation when compared to MyD88$^{-/-}$ allograft-infiltrating CD4$^+$ T cells from analogously treated recipients. Collectively, these results show that NET fragments utilize MyD88 signaling pathways to prevent immunosuppression-mediated acceptance of lung allografts.

Discussion

Here we show that the perioperative administration of DNAse after lung transplant primes innate immune responses that inhibit allograft survival. Notwithstanding this impact on tolerance DNAse improved early lung allograft function. The early beneficial effects of DNAse treatment have been reported in several models of sterile acute pulmonary injury including transfusion-related[8] and ventilator-induced acute lung injury[9]. Likewise, using a syngeneic mouse model of orthotopic lung transplantation, Sayah and colleagues recently demonstrated that the perioperative administration of DNAse improves graft function following ischemia-reperfusion injury[7]. Altogether these reports suggest that DNAse treatment is effective at improving lung function independently of the underlying etiopathogenesis of NET formation. Although the reasons for this are not clear, NETs have been shown to obstruct both small airways[34] and pulmonary vessels[8] suggesting that their physical removal improves lung ventilation and perfusion irrespective of the inflammatory consequences of their destruction.

DNAse treatment induced the release of NET fragments into the peripheral blood of lung transplant recipients. High circulating levels of NET fragments have been found in the plasma of patients affected by chronic inflammatory conditions such as Vasculitis[35] and Systemic Lupus Erythematosus[36]. In this regard, previous reports suggest that self-DNA-peptide circulating complexes may induce APCs maturation[37, 38]. Here we observed that NET fragments promote changes in APCs that potentiate alloimmunity. NET fragments stimulated human alveolar macrophages to express proinflammatory cytokines that promote CD4$^+$ T cell helper differentiation including IL-12, IL-6, IL-23 and IL-1β. In addition, NET fragments upregulated MHC II and costimulatory molecule expression on human and mouse dendritic cells. Notably, this effect was not completely abolished by complete digestion of NETs that non-DNA derived components of NET chromatin can also stimulate

inflammatory responses. Several reports have shown that NET-mediated tissue damage is mediated by histones, which act as damage associated molecular patterns that stimulate TLR2 and 4-mediated cytokine production[39, 40]. For example, in a model mouse transfusion induced acute lung injury - induced NETosis, the administration of antibodies to histones H2a and H4 inhibited lung edema[8]. Additionally, pretreatment of NET fragments with anti-histone Abs prevented endothelial cell damage[41]. Interestingly, in the latter report, DNAse treatment did not affect histone-mediated cytotoxicity raising the possibility that histones associated with DNA, as integrated within decondensed nucleosomes, are more inflammatory than free histones. However, it remains difficult to determine if this is the case as there is a lack of antibodies that can clearly distinguish between the two histone forms[42, 43]. Although in our study we did not directly address the role of histones, using TLR reporter lines we demonstrated that NET fragments elicited potent TLR2 and −4 activation that could mostly extinguished by complete digestion of NETs. We also observed that NET fragments stimulated TLR9 activation. Of note NETs have been recently demonstrated to contain mitochondria-derived hypo-methylated CpG DNA can activate TLR9[40]. Mitochondrial DNA can also stimulate inflammasome-dependent dendritic cell maturation through IL-1β- mediated MyD88 activation[44]. However, we observed wild-type like maturation in caspase1$^{-/-}$ dendritic cells suggesting that inflammasome activation is not critical for APC activation by DNAse. Additionally, and in line with the inability to activate TLR3, NET fragments stimulated the maturation of Trif$^{-/-}$ dendritic cells suggesting that the TLR-MyD88 independent pathway[45] does not control inflammatory responses driven by DNAse treatment.

We have previously demonstrated that unlike other solid organ transplants alloimmune responses to lung allografts are triggered by T cell priming within allograft tissue[29]. Noting that dendritic cells stimulated with NET fragments enhance CD4$^+$ T cell proliferation we asked if NET fragments drive early alloimmune responses within transplanted lungs. Relative to saline vehicle administration, DNAse or NET fragment treatment stimulated prolonged interactions between APCs and graft-infiltrating CD4$^+$ T cells one day after reperfusion. We also observed more intragraft effector CD4$^+$ T cells in DNAse or NET fragment-treated allografts. Because acute rejection is a risk factor for bronchiolitis obliterans syndrome (BOS), a form of chronic airway rejection that is the major limitation to the long-term graft survival[46], we also determined if DNAse or NET fragment administrations prevent lung allograft tolerance. DNAse or NET fragment administration promoted acute allograft rejection in B6 recipients immunosuppressed with cyclosporine and steroids. Importantly, DNAse administration to PAD4$^{\Delta/\Delta}$ recipients did not prevent immunosuppression-mediated lung allograft acceptance directly implicating the generation of NET fragments in triggering allograft rejection. MyD88 expression within the donor allograft cells was required for allograft rejection along with the accumulation of intragraft alloantigen specific T_h1 and T_h17 effector cells. Although MyD88 is likely expressed in all pulmonary cells APCs predominantly utilize this adaptor molecule to primarily promote inflammatory gene expression while parenchymal cells depend use it to promote survival and repair[47, 48]. Taken together with our observations of NET fragment-mediated interactions between donor-derived CD11c$^+$ APCs and CD4$^+$ T cells our data suggest that

TLR-MyD88 signaling within the donor-derived APC compartment is required to promote DNAse-mediated alloimmune responses that prevent tolerance.

Finally, we also detected $CD4^+$ T cell responses directed against the pulmonary self-antigens Collagen V and K-α1 tubulin. Consistent with observations made in rejecting human lung recipients[49], many of these autoreactive $CD4^+$ T cells expressed IL-17A. As T_h17 cells drive neutrophilia through stimulating the expression of ELR^+ CXC chemokines and granulopoietic cytokines[50] an unintended downstream consequence of NET fragment generation could be the promotion of neutrophil-mediated digestion of pulmonary tissue and the release of autoantigens. In conclusion, we have demonstrated that DNAse treatment stimulates innate immune responses within lung allografts that promote alloimmunity. Future investigation may be warranted into other approaches that prevent NETosis in transplant organs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APC	antigen presenting cell
BAL	bronchoalveolar lavage
Balb. WT	Balb/c wild-type
Balb.MyD88−/−	Balb/c MyD88−/− deficient
BMDC	bone marrow-derived dendritic cells
BOS	bronchiolitis obliterans syndrome
CF	Cystic Fibrosis
cit-H3	citrullinated histone 3
ColV	Collagen V
iSTAT	iSTAT Portable Clinical Analyzer
Kα1T	K-α1 tubulin
MPO	myeloperoxidase
MyD88	Myeloid Differentiation Primary Response 88
NET Sup	supernatents from PMA-stimulated NETs

NETfrag	NET fragments
NETs	Neutrophil extracellular traps
NETsext	Completely digested NETs
NETsonic	sonically sheared NETs
PAD4	Protein Arginine Deiminase 4
PGD	primary lung graft dysfunction
POD	post-operative day
TLR	Toll-like receptor

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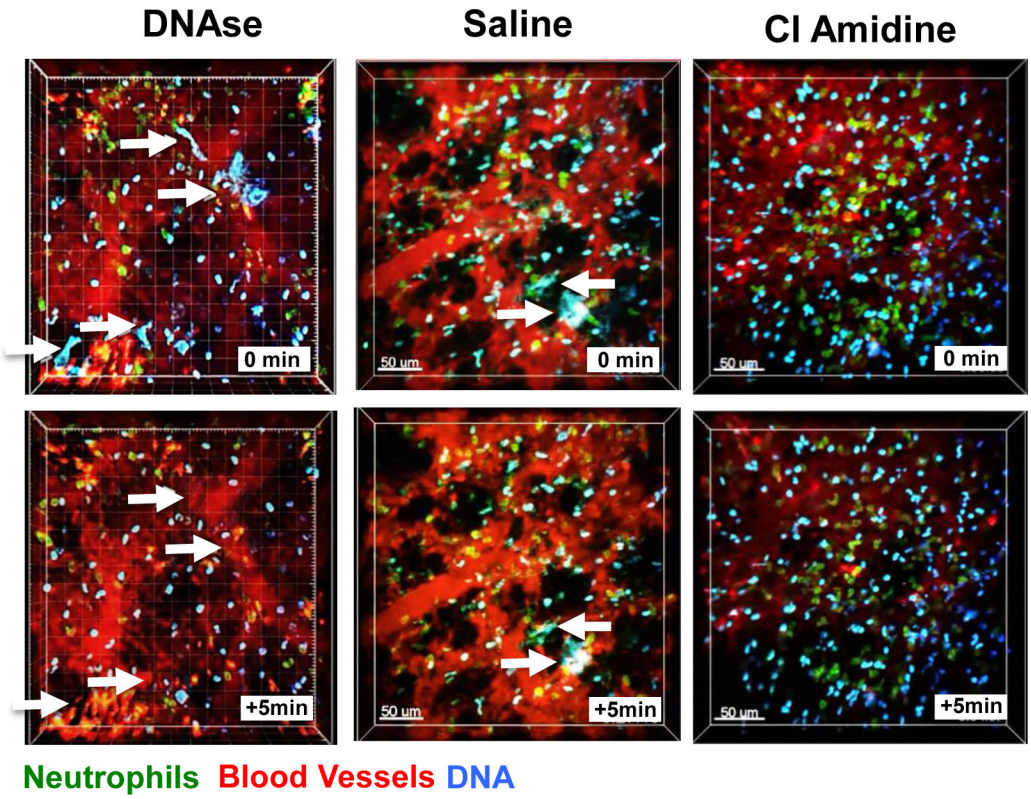

Figure 1: Intravital imaging of lung allografts treated with DNAse.

Representative intravital images of ischemically-injured Balb/c → B6 lung recipients just prior to or 5 minutes after DNAse or saline vehicle treatment or ischemically-injured Balb/c → B6 lung recipients pretreated with Cl-Amidine and observed over an analogous 5 min period (N = 4 / group) approximately 30 minutes after reperfusion. White arrows denote NETs or positions of NETs after fragmentation.

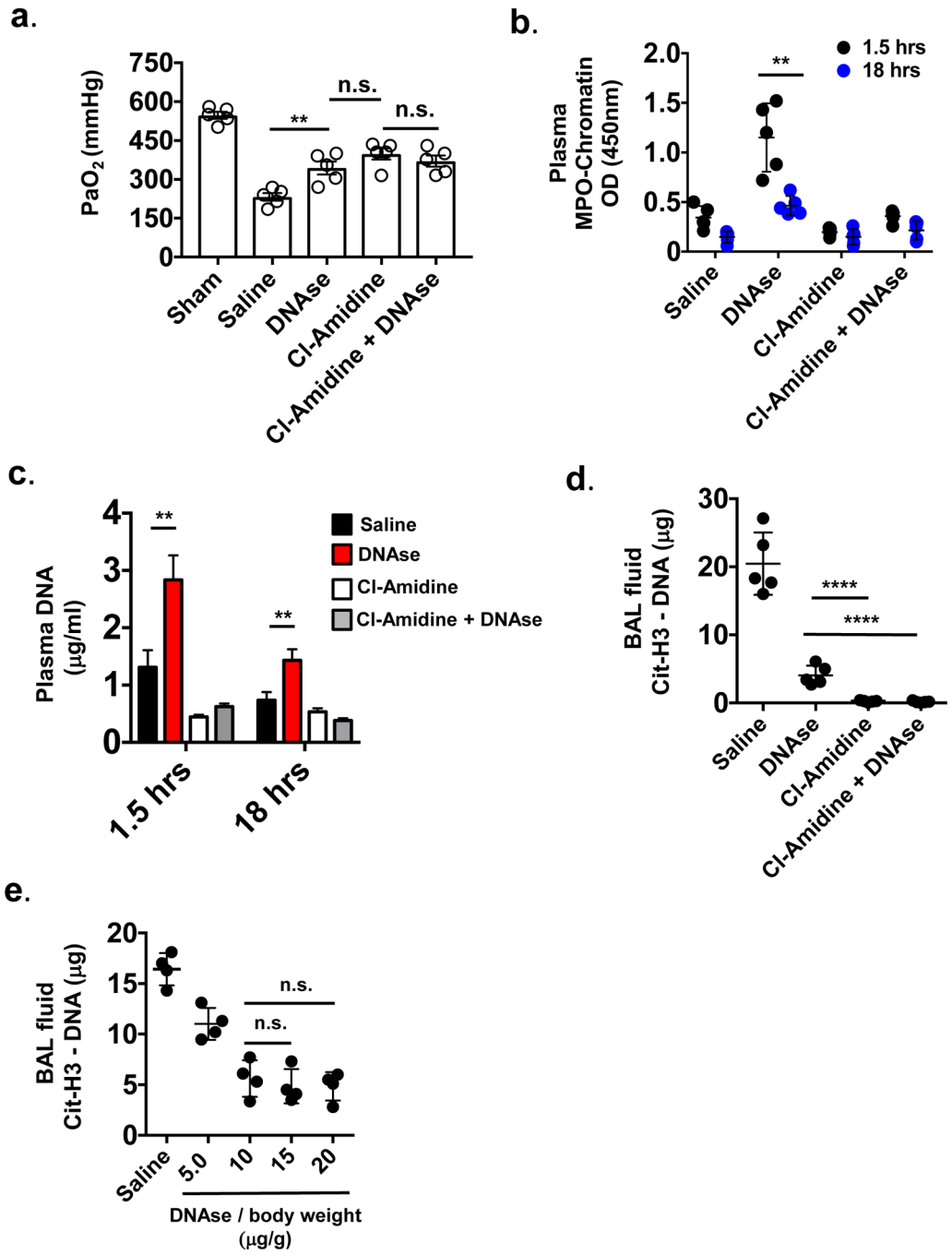

Figure 2: Following DNAse treatment NET fragments are released into the peripheral circulation.

(a) Sham operated B6 mice or ischemically-injured Balb/c → B6 lung recipients following indicated treatment (N=5 / group) were assesed for lung function at 90 minutes after reperfusion. Ischemically-injured Balb/c → B6 lung recipients following indicated treatments were assed for NETs within circulating plasma as measured by **(b)** ELISA-based detection of MPO associated DNA complexes and (**c**) total double stranded DNA quantitation following treatment with saline, DNAse, Cl-Amidine or Cl-Amidine & DNAse.

(d) Balb/c→ B6 lung recipients (N=5 / group) BALF 1.5 hours after reperfusion were quantified by ELISA for citrullinated histone (Cit-H3b) associated DNA complexes 1.5 hours after engraftment. **(e)** B6 mice following clamping of the right hilum to induce left lung warm ischemic injury received either saline or indicated doses of DNAse 30 minutes after reperfusion. 1.5 hours after hilar clamping BAL fluid was collected and analyzed for cit-H3b DNA complexes. Where indicated means with ± standard deviation (S.D.) are shown with n.s as non-significant, **p< 0.01 and ****p <0.0001.

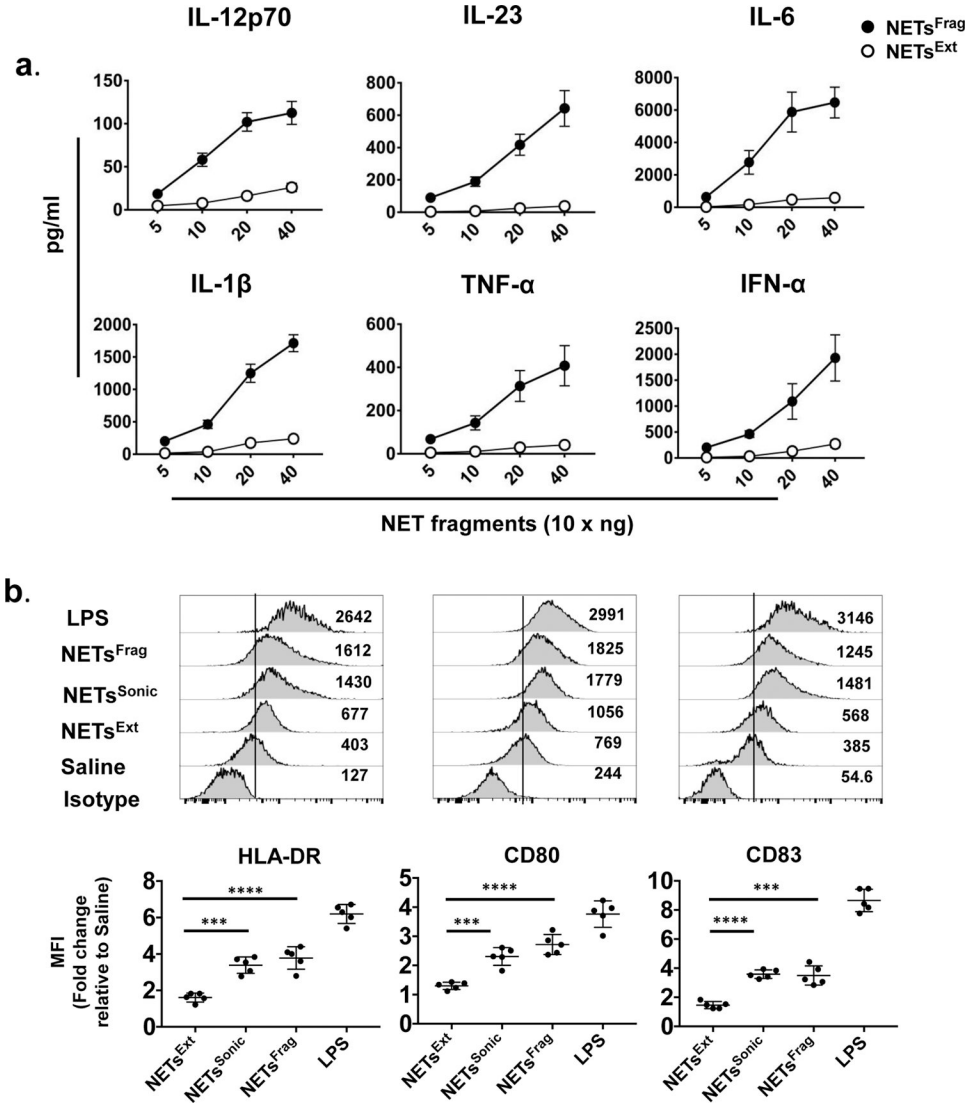

Figure 3: NET fragments promote inflammatory gene expression in human alveolar macrophages and the maturation of human monocyte-derived dendritic cells.

(**a**) Alveolar macrophages isolated from human donor lungs were stimulated with indicated amounts of human NET fragments pooled from DNAse treated NETs from at least 10 healthy volunteers. 18 hours later cultures were assessed for cytokine production by bead array. Data shown are representative results from 3 independent experiments where cytokine production is shown as a mean ± S.E.M from alveolar macrophage cultures from three donors. **(b)** Human peripheral blood monocyte derived dendritic cells (HMDC) were assessed for HLA-DR (MHC II), CD80 and CD83 expression 24 hours after being stimulated with LPS (100ng/ml), DNAse fragments (1 μg/ml, $NETs^{Frag}$), sonicated NETs (1 μg/ml, $NETs^{Sonic}$), overnight DNAse digested NETs (1 μg/ml equivalent of $NETs^{Frag}$; $NETs^{Ext}$) or saline. Data in the top panel are representative results from an HMDC culture generated from (bottom panel) one of five volunteers. Data shown in the lower panel are responses from individual HMDC donors and also represented as a group mean ± S.D., where ***p< 0.001 and ****p <0.0001.

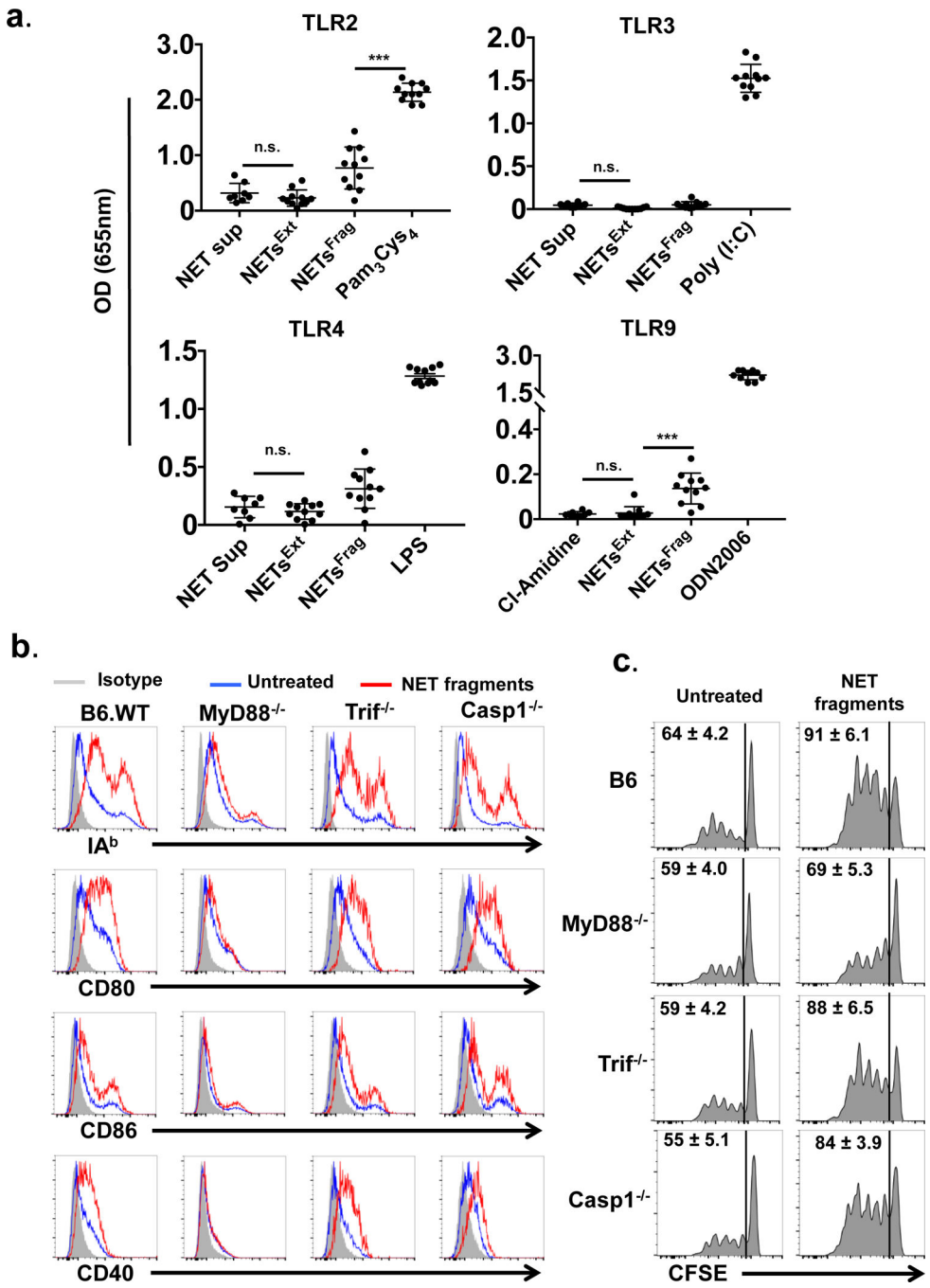

Figure 4: NET fragments stimulate mouse dendritic cell maturation and CD4^{+}T cell proliferation through TLR-MyD88 signaling pathways.

(a) Indicated TLR reporter lines were cultured with enriched supernatant from PMA-stimulated human neutrophils (NET Sup) at 25% v/v, NETs^{Frag} (1 μg/ml), NETs^{Ext} (1 μg/ml equivalent) and respective positive controls Pam_3Cys_4 (1 μg/ml), Poly I:C (1 μg/ml), LPS (100ng/ml) and ODN2006 (5 μM). Each data point represents TLR activation from NETs fragments from a single volunteer where the overall response from 11 individuals is shown as a mean ± S.D. ***$p < 0.0001$. **(b)** B6, MyD88$^{-/-}$, Trif$^{-/-}$ and Casp1$^{-/-}$ bone marrow

derived dendritic cells stimulated with NET fragments for 24 hours and assessed for IA^b (MHC II), CD80, CD86 and CD40 expression or **(c)** the ability to drive allogeneic (Balb. WT) $CD4^+$ T cells proliferation as measured by CFSE dilution. Data shown in **(b)** are representative of 3 independent experiments. Proliferation for each group in **(c)** (N=3/group) is shown as mean CFSE dilution ± S.D.

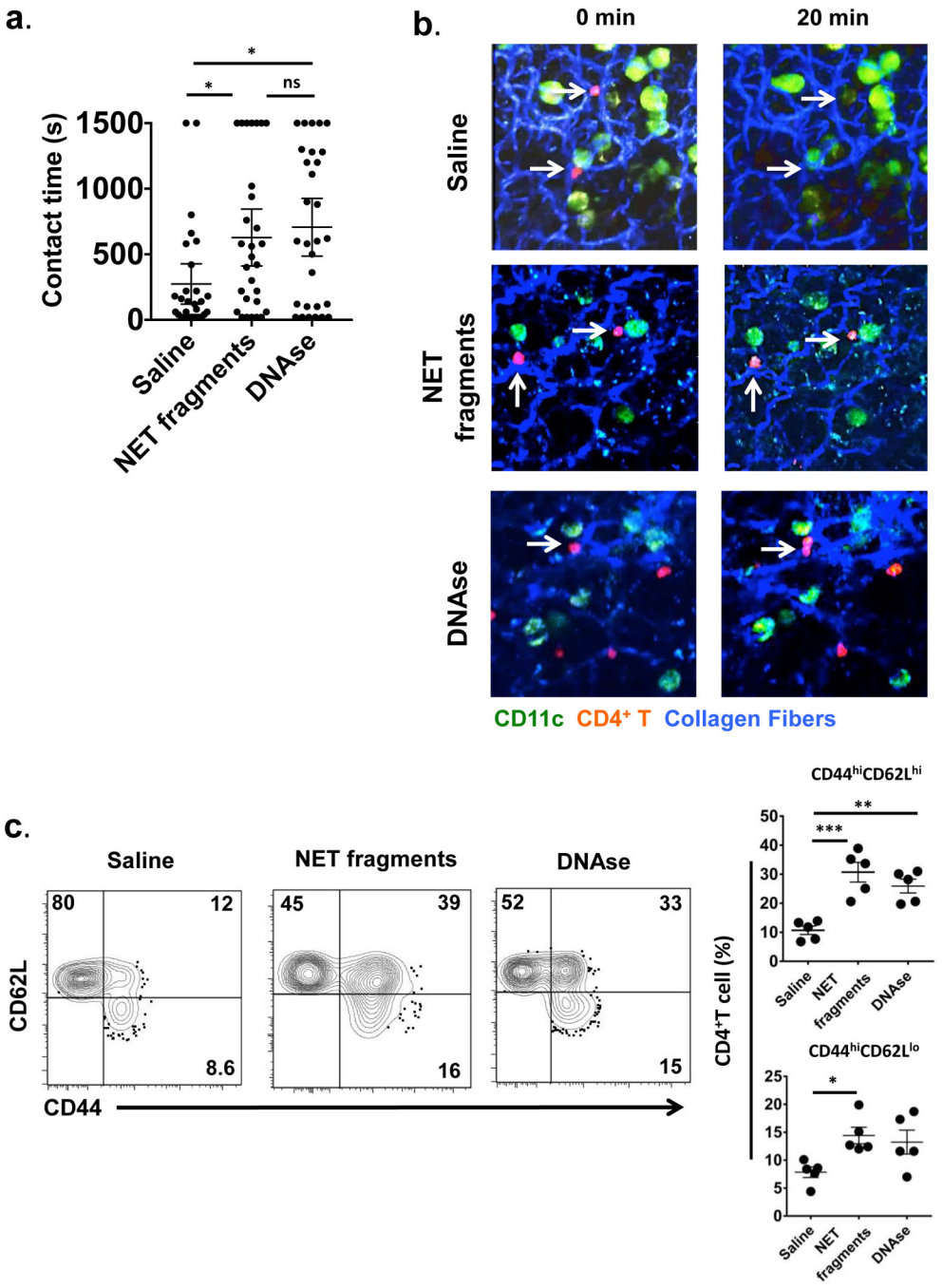

Figure 5: NET fragments induce naïve CD4$^+$ T cell activation within lung allografts.

CD11c EGFP recipients were transplanted ischemically-injured Balb/c lung recipients received saline, DNAse or NET fragments, along with received 10^7 naïve CMTMR tracking dye - labeled Balb.WT CD4$^+$ T cells (Red) and 24 hours later were intravitally imaged for association with donor-derived CD11c$^+$ cells (Green). **(a)** Each data point represents a single association lasting from 15 to 1500 seconds from at least four transplants. **(b)** Representative images from **(a)**. White arrows denote CD4$^+$ T-CD11c$^+$ cell associations over an approximate 20 min interval. **(c)** B6 recipients of ischemically-injured Balb.WT lungs

received treatments as in **(a)** along with 5×10^6 naïve B6 CD45.1$^+$ CD4$^+$ T cells that were assessed for differential changes in CD44 and CD62L expression 48 hours after adoptive transfer. Contour plots show a representative result from each treatment group where expression is depicted as a mean ± S.E.M; **p < 0.01, ***p < 0.005.

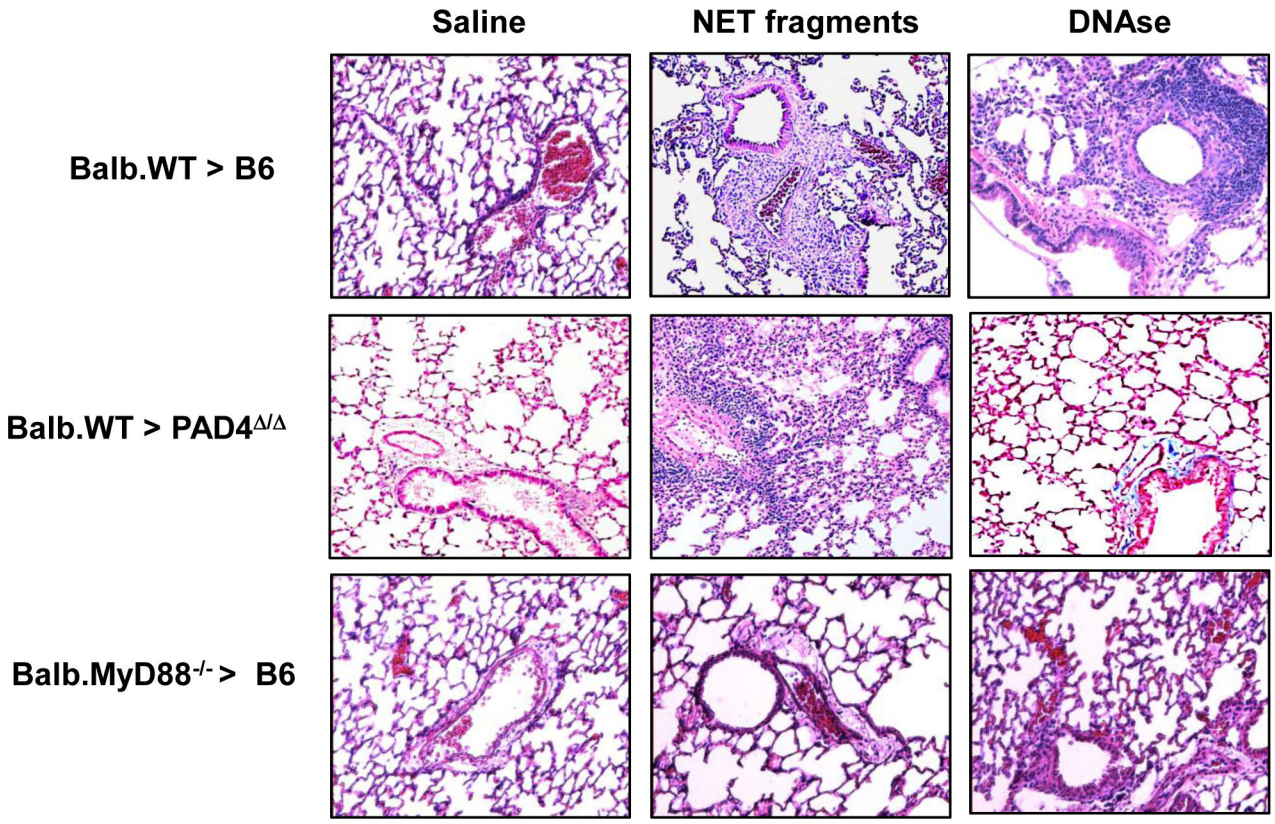

Figure 6: NET fragments promote acute vascular lung allograft rejection

Ischemically-injured Balb.WT lungs were transplanted into B6 or PAD4$^{\Delta/\Delta}$ recipients or ischemically-injured Balb/c. MyD88$^{-/-}$ lungs were transplanted into B6 recipients. Recipients received DNAse (10μg/g body weight), intratracheal NET fragments (2.5 μg) or saline vehicle 30 minutes after reperfusion along with daily immunosuppression consisting of cyclosporine A (10 mg/kg body weight /day) and methylprednisolone (1.6 mg/kg body weight /day). On POD 7 lung allografts were analyzed for vascular rejection by H&E histology. Data shown are representative histology from one of five transplants.

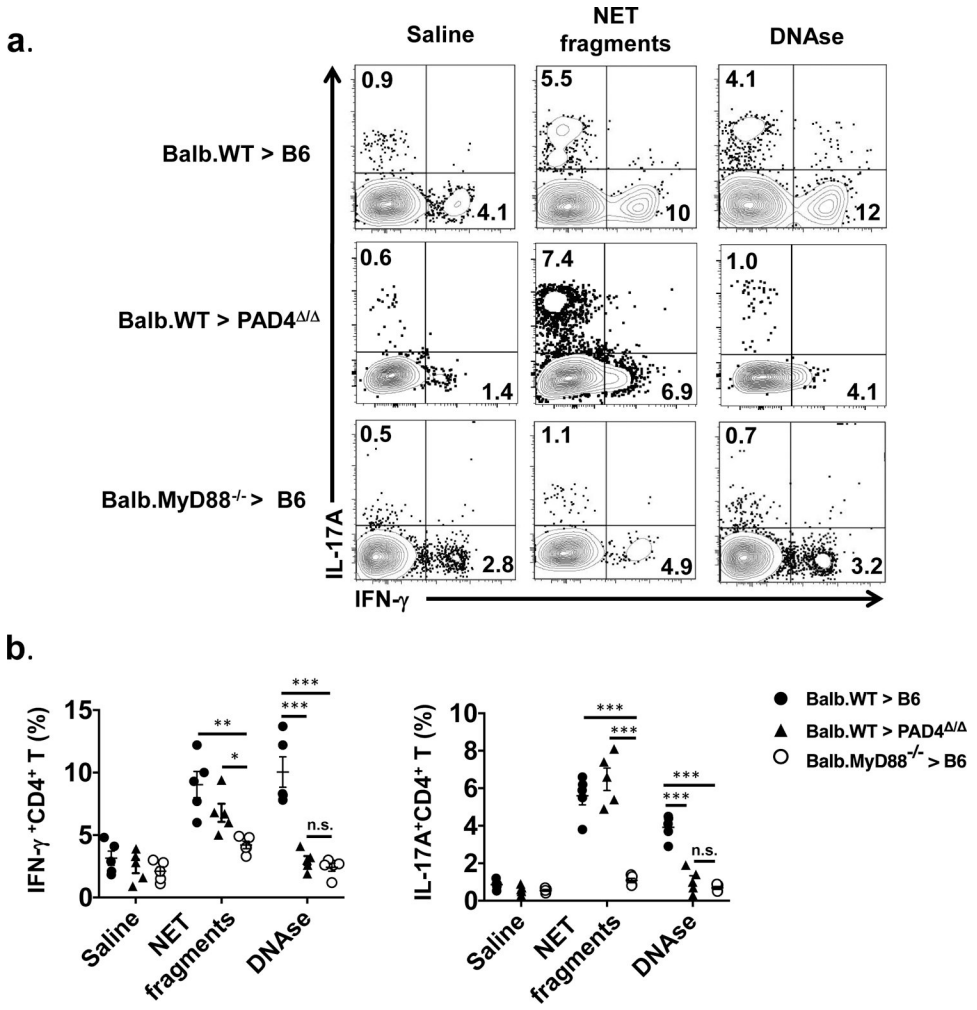

Figure 7: NET fragments stimulate intragraft accumulation of effector T cells.

Ischemically-injured Balb.WT lungs were transplanted into immunosuppressed B6 or PAD4$^{\Delta/\Delta}$ recipients or ischemically-injured Balb. MyD88$^{-/-}$ lungs were transplanted into immunosuppressed B6 recipients. Recipients received DNAse (10 μg/g body weight), intratracheal NET fragments (2.5 μg) or saline vehicle 30 minutes after reperfusion. On POD 7 lung allografts were assessed for percent abundance of IFN-γ^+ CD4$^+$ and IL-17A$^+$ CD4$^+$ T cells. Data shown in **(a)** are representative contour plots from one of five transplants. Plots in **(b)** show percent abundance from individual transplants with a mean ± S.D. where *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

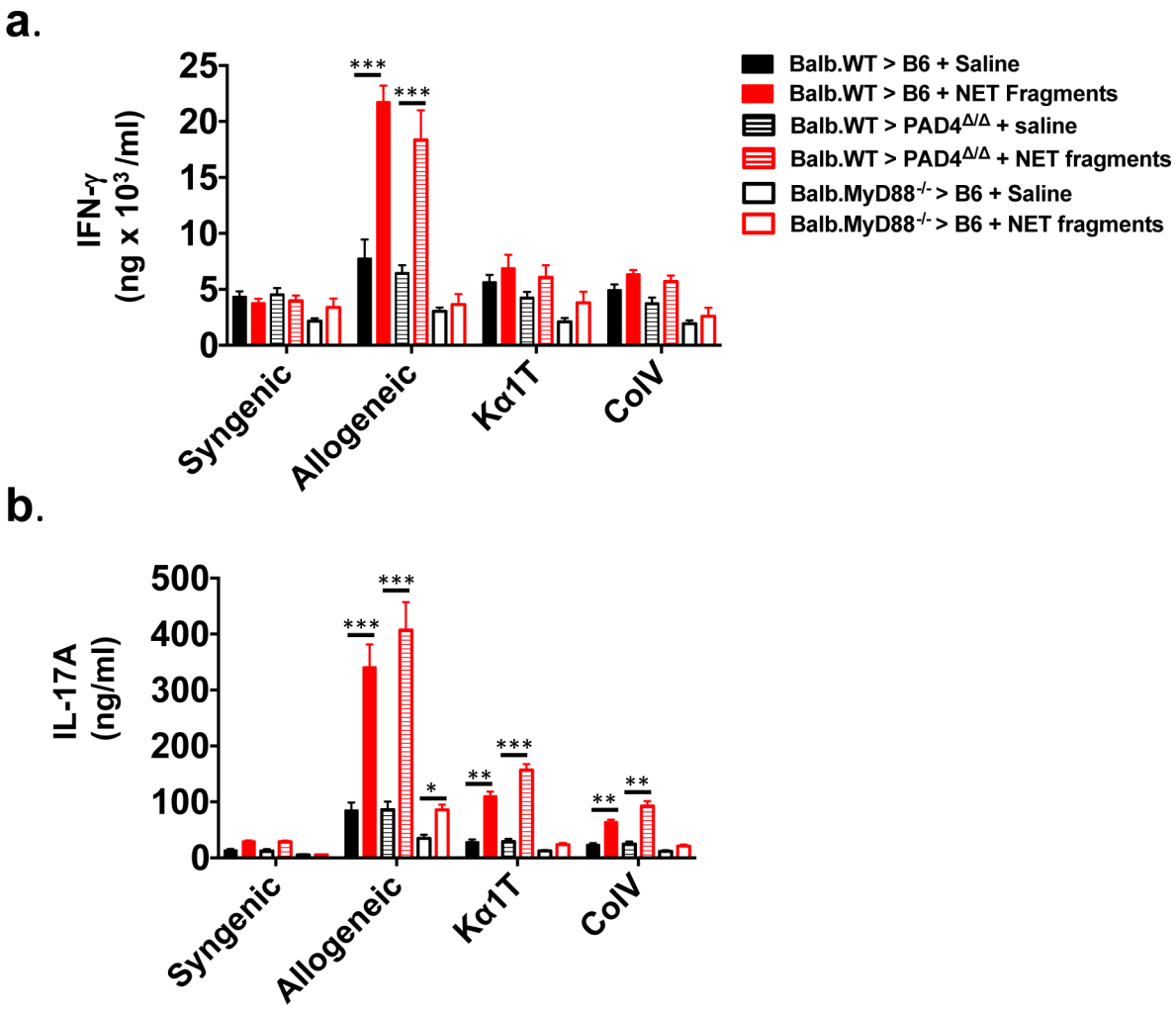

Figure 8: NET fragments prevent tolerance to allo- and self-antigens.

Ischemically-injured Balb.WT lungs were transplanted into immunosuppressed B6 or PAD4$^{\Delta/\Delta}$ recipients or ischemically-injured Balb. MyD88$^{-/-}$ lungs were transplanted into immunosuppressed B6 recipients. On POD 7 intragraft CD4^{+} T cells were fractionated from allografts, co-cultured with T-cell depleted Balb/c splenocytes or B6 splenocytes with or without Kα1T or ColV peptides and then assessed for **(a)** IFN-γ and **(b)** IL-17A production 96 hours later. Data shown are from 5 transplants per group where expression is depicted as a mean ± S.D.; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.