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# **Neutrophil extracellular traps and DNase I levels in the bronchoalveolar lavage fluid of children with pulmonary injury after allogeneic hematopoietic stem cell transplantation: a TRANSPIRE study**

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**Running head:** NETs and DNase I levels in BAL fluid after pediatric HSCT

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Twenty-five to thirty percent of patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) will experience pulmonary complications, which are a major cause of non-relapse mortality and morbidity <sup>1,2</sup>. The underlying mechanism of tissue injury is variable, depending on the pathology, and hypothesized to be related to conditioning toxicity, early endothelial injury, infections leading to direct and indirect tissue injury and immune responses leading to interstitial lung disease, or graft versus host disease (GVHD). There is a crucial need to identify targets for drug development to limit tissue injury during transplant and at the time of acute pulmonary events to prevent irreversible pulmonary injury and mortality.

Neutrophils are the first cells to arrive at any site of tissue injury. Gassas et al showed that children who developed bronchiolitis obliterans syndrome (BOS) had significantly higher neutrophil counts at three and six months post-HSCT compared to children who did not develop BOS <sup>3</sup>. Neutrophils use multiple mechanisms to enhance host defense, including formation of neutrophil extracellular traps (NETs) <sup>4</sup>. Dysregulation in clearance of NETs is a known cause of severe lung injury in patients with severe infections, ARDS, and lung transplantation <sup>5-7</sup>. Previous studies from our group have shown that levels of NETs are associated with HSCT complications like endothelial injury, including lung injury and GVHD <sup>8,9</sup>. Cell free DNA released at the time of NETosis is removed by endogenous defense mechanisms like DNases, which are also variable in patients undergoing HSCT <sup>10</sup>. Recombinant inhaled DNase is a possible targeted agent against NETs, but there are no data on local levels of NETs or DNases in the lungs of patients undergoing HSCT.

We collected bronchoalveolar lavage fluid (BALF) and plasma samples at the time of a pulmonary event from patients who were enrolled in the Cincinnati Children's Hospital Medical Center HSCT biorepository and the TRANSPIRE study between 2019 to 2025. The TRANSPIRE study (NCT04098445) is a multi-institutional prospective cohort study of pediatric and young adult HSCT recipients including eight US pediatric transplant programs. Bronchoscopy was performed for clinical care and performed according to clinical practice at each institution. During bronchoscopy, 5-10ml of BALF were reserved for research purposes and was stored at 4°C until processing. For processing, the BALF was centrifuged twice and was aliquoted with protease and phosphatase inhibitors (Roche 04693124001, Roche 04906845001). The aliquots were stored at -80°C for future analysis. We reviewed medical records, including cytology reports from BALF, respiratory symptoms, changes in FEV1%, and chest imaging for BOS assessment per American Thoracic Society (ATS) guidelines <sup>11</sup>. If patients had more than one bronchoscopy performed for the same event, only the first bronchoscopy data and BALF was studied. We classified the events leading to bronchoscopy into 8 groups - pre-transplant/screening, infection, endothelial injury, diffuse alveolar hemorrhage (DAH), idiopathic pulmonary syndrome (IPS), early BOS - patients who met ATS BOS criteria within 6 months of BALF sample was collection (bronchoscopy), late BOS - patients who met ATS criteria at least 6 months before BALF sample was obtained, and BOS with infection. Samples that were positive for infection in the pre-transplant/screening bronchoscopy were categorized as infection. We measured circulating double stranded DNA (ds-DNA) as a surrogate of NETs (Quant-iT PicoGreen (P7589; Molecular Probes)), DNase I (MyBioSource MBS2515385) and monocyte chemoattractant protein-1 (MCP-1)

(DCP00 from R&D Systems) by ELISA in plasma and BALF as previously described <sup>12</sup>. This study was conducted in accordance with the principles outlined in the Declaration of Helsinki and was approved by the Institutional Review Board.

We studied total of 119 BALF samples, collected from 96 patients. Patient demographics are shown in Table 1. Median age at the time of HSCT was 11 years (4 months – 38 years). A majority of patients were male (64%), Caucasian (84%), had an underlying diagnosis of malignancy (55%), and received a matched unrelated donor (MUD) (53%) transplant with a bone marrow graft (60%). Most patients received calcineurin based GVHD prophylaxis with (38%) or without abatacept (46%).

Median time from transplant to bronchoscopy was 70 days (days -118 to 3137) (Supplemental Table 1). Twenty-four percent of BALF samples (n=28) were obtained for pre-transplant or screening evaluation without any respiratory symptoms and served as a control for cases with acute pulmonary events. Thirty-four percent of the BALF obtained at the time of a pulmonary event resulted in diagnosis of infection (n=40, bacterial: 45% n=18, viral: 45%, n=18, fungal: 5%, n=3). Twenty seven percent (n=32) of pulmonary events were in patients with BOS - 11 with early BOS, 8 patients with late BOS, and 15 from patients who had BOS complicated by infection. Five patients were diagnosed with endothelial injury syndromes, 8 with IPS and 3 with DAH.

We first measured dsDNA in BALF. Baseline levels of dsDNA in pretransplant/screening patients were low at median of 13.8 ng/ml (IQR: 11.0-18.0 ng/ml), while levels in post-transplant BALF samples at the time of pulmonary event were significantly higher (median 298.5 ng/ml, IQR: 63.7-541.9 ng/ml) (Fig 1A). Figure 1B displays dsDNA levels according to the pulmonary injury incurred. dsDNA levels were generally higher in

patients in whom infection was diagnosed by the bronchoscopy, including patients with BOS and infection and those with infection alone. Patients with late BOS had low levels of dsDNA, while levels were higher in those with early BOS (392.3 ng/ml (IQR: 19.2-580.4 ng/ml) vs 63.7 ng/ml (IQR: 25.4-96.8 ng/ml)), suggesting a possible inflammatory neutrophilic component in early BOS <sup>3</sup>. These data are summarized in supplementary Table 1. The proportion of neutrophils identified in BALF by conventional clinical cytology was positively correlated with dsDNA ( $r=0.67$ ,  $p<0.0001$ ), Fig 1C. Patients with late BOS had relatively lower levels of dsDNA, despite presence of neutrophils, Fig 1D. It is also likely that a cytological percentage alone is a poor reflection of the total number of neutrophils in the airways.

Patients with IPS had relatively lower levels of dsDNA, suggesting that neutrophils might not be the main cells playing a role in pathophysiology of IPS. The pathophysiology of IPS after HSCT is poorly characterized, but a previous study has described a possible role for MCP-1 in IPS after HSCT <sup>13</sup>. We measured MCP-1 levels in a subset of patients and found that MCP-1 levels are significantly higher in patients with IPS confirming the findings of Gerhard et al (Supplementary Fig 1A, 1B). These data support the concept that IPS might be a monocyte/macrophage driven disease.

We next measured levels of DNase I. DNase I levels in BALF collected before transplant in 28 patients were low at 8.1 ng/mL (IQR: 4.5-26.4), while levels in post-transplant levels were significantly higher (median 24.1 ng/mL, IQR: 14.2-40.2) Fig 2A. Figure 2B show DNase I levels according to diagnosis at the time of bronchoscopy. There was a wide distribution of DNase I levels in patients with infection. DNase I is ubiquitously expressed and is a highly inducible gene. BALF dsDNA and BALF DNase I

levels were positively correlated,  $r = 0.49$ ,  $p = < 0.0001$  (Fig 2C). However, some patients had lower than median levels of DNase I levels despite high dsDNA in BALF, Fig 2C. Forty-eight post-transplant samples had BALF dsDNA above the median, but 18 of those samples had a DNase I level below the median suggesting a potential group of patients who might benefit from localized intervention with inhaled DNase I.

We then considered whether the information gathered by directly measuring BALF dsDNA and DNase I levels might be more easily obtained using laboratory tests of peripheral blood or more conventional evaluations of BALF. We found no correlation between plasma dsDNA and BALF dsDNA (Supplementary Table 2). We also examined absolute neutrophil count (ANC), BALF dsDNA and neutrophils and found weak correlations (Supplementary Table 2). Similarly, BALF DNase I and plasma DNase I were not correlated. These data support a degree of pulmonary compartmentalization of inflammation during acute lung injury after HSCT, suggesting potential benefit of bronchoscopy and direct measurement of dsDNA in BALF if there is consideration of targeted therapy <sup>14, 15</sup>.

In conclusion, these data provide information supporting consideration of bronchoscopy and BALF evaluation for biomarkers of NETosis and DNase I in patients with pulmonary complications after HSCT.

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**Table 1: Demographics**

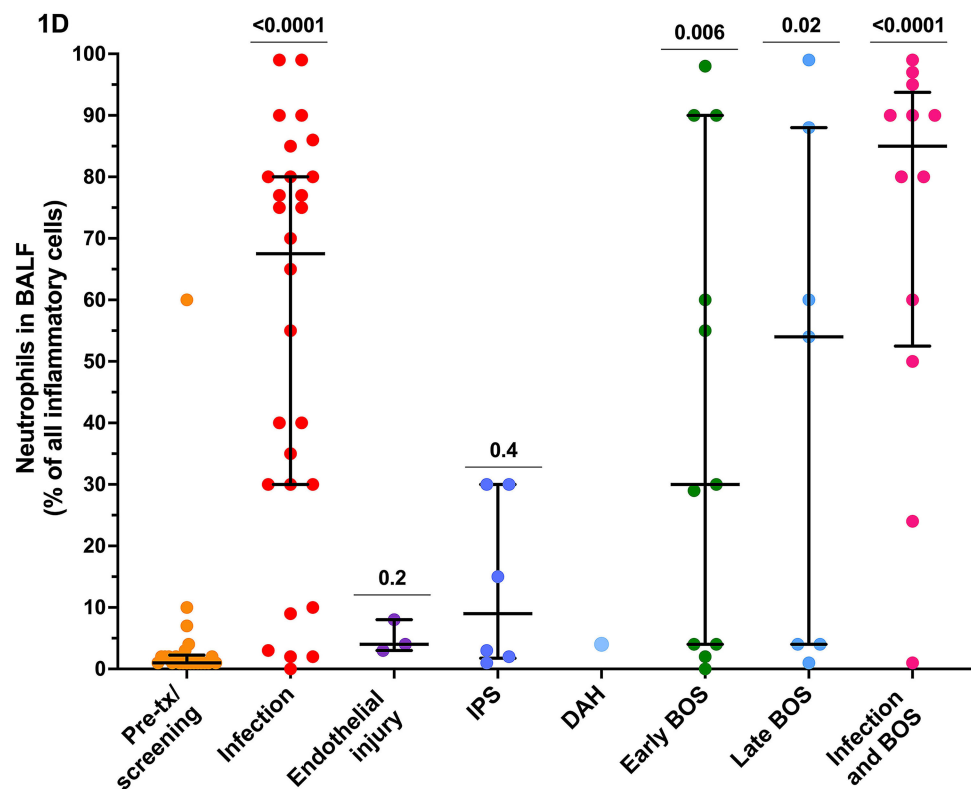
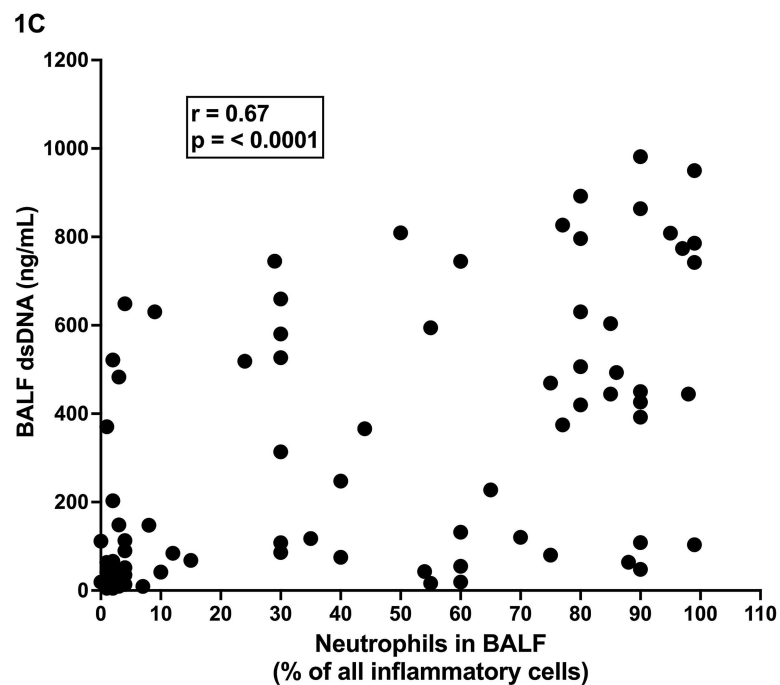
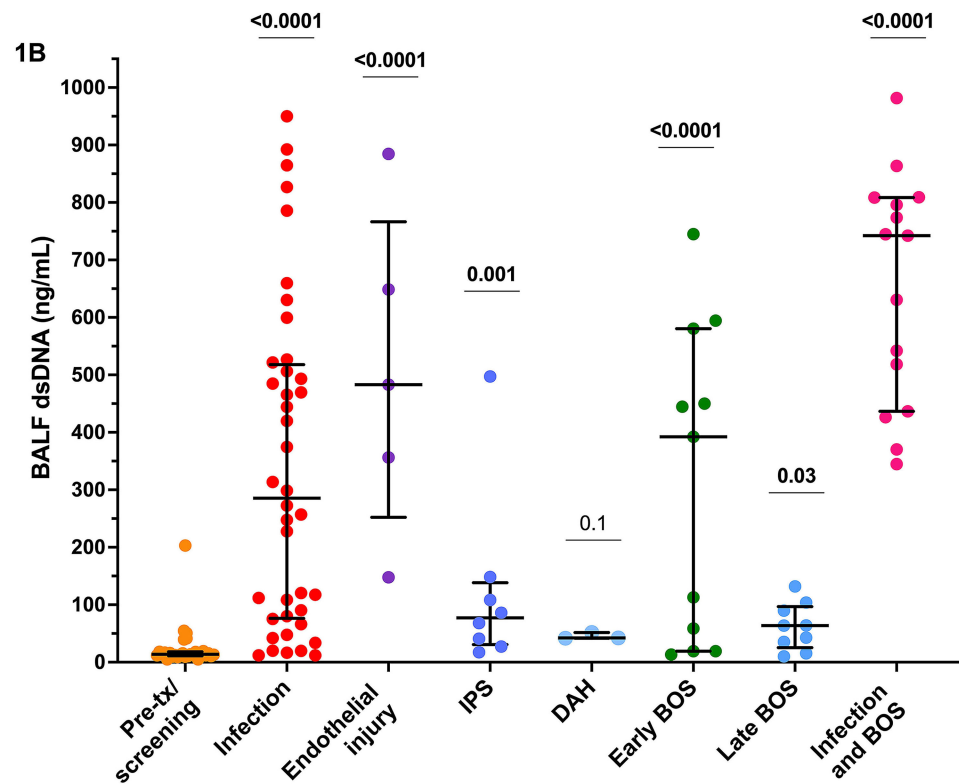
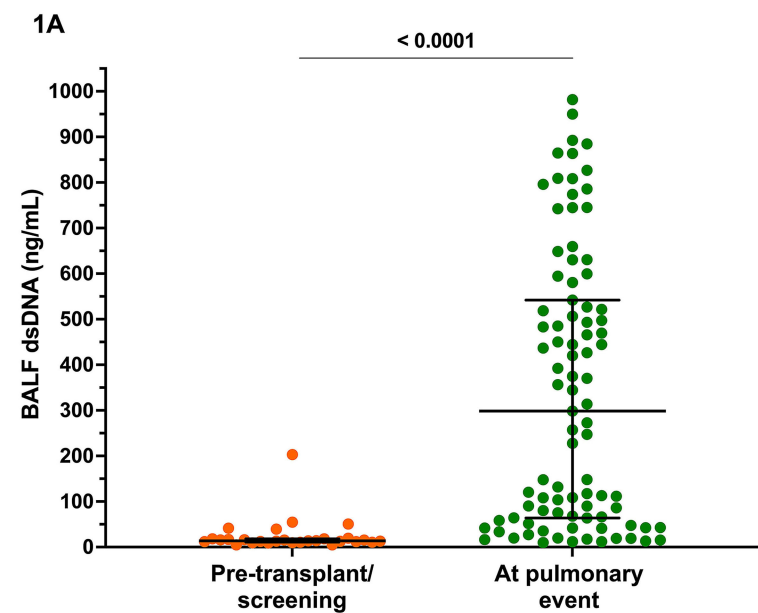
| <b>Demographics</b>              | <b>n=96</b>                    |
|----------------------------------|--------------------------------|
| <b>Age (median, range)</b>       | 11 years (4 months – 38 years) |
| <b>Gender (n)</b>                |                                |
| Male                             | 61 (64%)                       |
| Female                           | 35 (36%)                       |
| <b>Race</b>                      |                                |
| Caucasian, n (%)                 | 80 (84%)                       |
| AA, n (%)                        | 8 (8%)                         |
| Asian, n (%)                     | 5 (5%)                         |
| Caucasian/AA, n (%)              | 2 (2%)                         |
| Unknown, n (%)                   | 1 (1%)                         |
| <b>Diagnosis</b>                 |                                |
| Malignancy, n (%)                | 52 (55%)                       |
| Immune deficiency, n (%)         | 24 (26%)                       |
| Marrow failure, n (%)            | 14 (15%)                       |
| Hematologic disorder, n (%)      | 4 (4%)                         |
| Genetic disorder, n (%)          | 2 (2%)                         |
| <b>Donor type</b>                |                                |
| Unrelated, n (%)                 | 70 (73%)                       |
| Related, n (%)                   | 26 (27%)                       |
| <b>HLA type</b>                  |                                |
| Matched, n (%)                   | 51 (53%)                       |
| Mismatched, n (%)                | 36 (38%)                       |
| Haploidentical, n (%)            | 9 (9%)                         |
| <b>Stem cell source</b>          |                                |
| BM, n (%)                        | 58 (60%)                       |
| PBSC, n (%)                      | 23 (24%)                       |
| Cord, n (%)                      | 15 (16%)                       |
| <b>Conditioning regimen type</b> |                                |
| MAC, n (%)                       | 67 (70%)                       |
| RIC, n (%)                       | 28 (29%)                       |
| None, n (%)                      | 1 (1%)                         |
| <b>GVHD prophylaxis</b>          |                                |
| CNI based, n (%)                 | 44 (46%)                       |
| CNI with abatacept, n (%)        | 36 (38%)                       |
| T cell depletion, n (%)          | 8 (8%)                         |
| Pt-Cy, n (%)                     | 7 (7%)                         |
| None, n (%)                      | 1 (1%)                         |

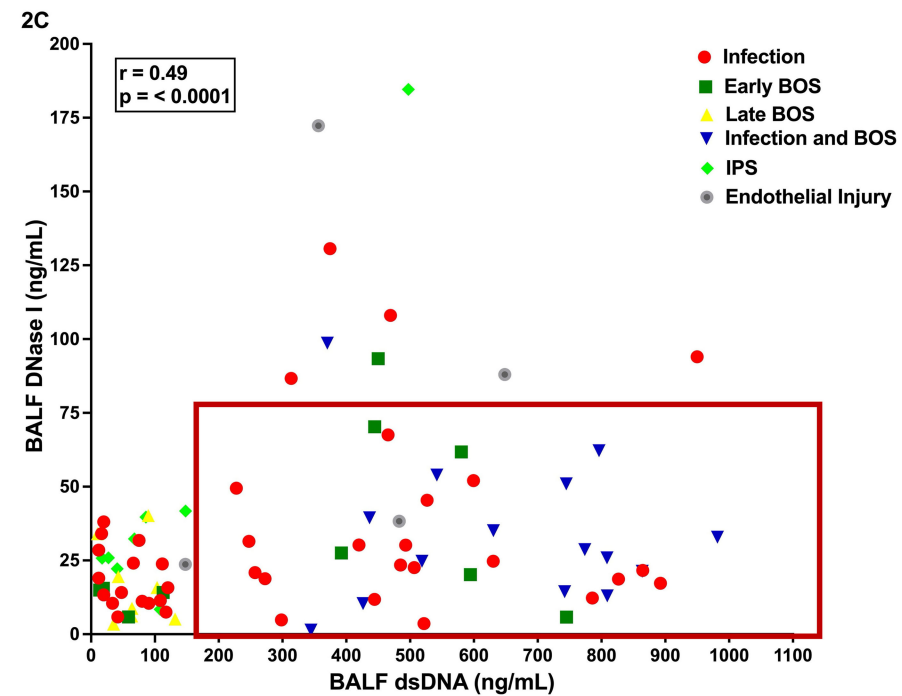
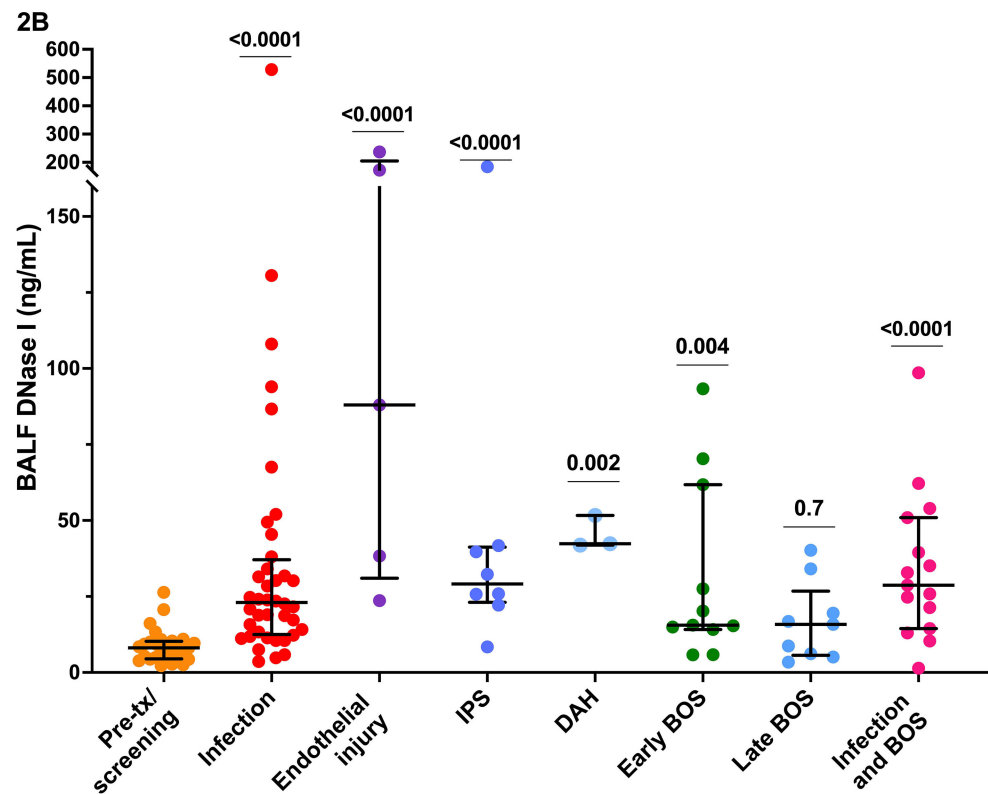
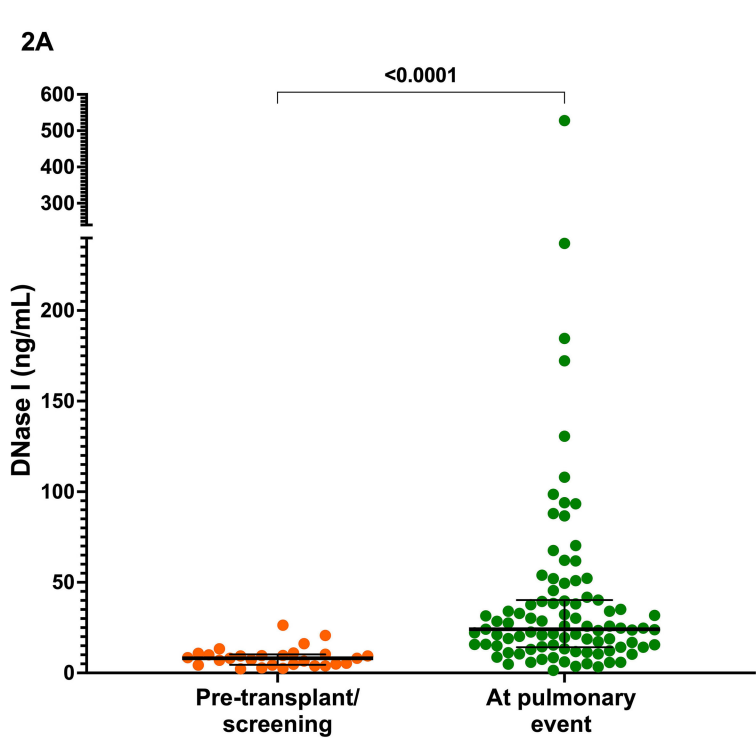
AA: African American, BM: Bone marrow, PBSC: Peripheral Blood Stem Cells, Pt-Cy: Post-transplant Cyclophosphamide, CB: cord blood, MAC: myeloablative conditioning, RIC: reduced intensity conditioning, CNI: Calcineurin inhibitor.

## Figure legends:

**Figure 1. dsDNA levels and neutrophil percentage in bronchoalveolar lavage fluid (BALF).** **A.** BALF dsDNA levels pre-transplant compared to post-transplant (median, IQR). **B.** BALF dsDNA levels at the time of bronchoscopy per diagnosis (p-values were generated in comparison to pre-tx/routine group using Mann Whitney U test and adjusted for multiplicity using a Bonferroni correction). **C.** Correlation between BALF dsDNA and BALF neutrophil percentage by cytology (reported as % of all inflammatory cells) (calculated by Spearman Correlation) **D.** Neutrophils in BALF reported as % of all inflammatory cells (p-values were generated in comparison to pre-tx/routine group using Mann Whitney U test and adjusted for multiplicity using a Bonferroni correction)

**Figure 2. DNase I levels in bronchoalveolar lavage fluid (BALF) and their correlation with dsDNA in BALF.** **A.** BALF DNase I levels pre-transplant compared to post-transplant (median, IQR). **B.** BALF DNase I levels at the time of bronchoscopy per diagnosis (p-values were generated in comparison to pre-tx/routine group using Mann Whitney U test and adjusted for multiplicity using a Bonferroni correction). **C.** Correlation between BALF dsDNA and BALF DNase I (spearman correlation). Red box: samples with high dsDNA but low DNase I, suggesting some patients may be candidates for consideration of inhaled DNase I as a targeted therapeutic agent.





Supplemental tables:

**Supplemental Table 1:** Diagnosis, time to pulmonary event (days post-transplant, median, range) and levels of NETs, DNase I and Neutrophil percentage in BALF (median, interquartile range)

|                                                | Pre-transplant/<br>screening<br>(n=28) | Infection<br>(n=40)            | EI<br>(n=5)                   | IPS<br>(n=8)                  | DAH<br>(n=3)        | Early BOS<br>(n=11)           | Late BOS<br>(n=9)          | Infection<br>and BOS***<br>(n=15) | p-value** |
|------------------------------------------------|----------------------------------------|--------------------------------|-------------------------------|-------------------------------|---------------------|-------------------------------|----------------------------|-----------------------------------|-----------|
| <b>Days post-transplant</b><br>(median, range) | (-21)<br>(-71 – 211)                   | 77.5<br>(-118 – 2440)          | 18<br>(13 – 229)              | 36.5<br>(16 – 235)            | 132<br>(54-210)     | 124<br>(34 – 483)             | 666<br>(106- 2698)         | 914<br>(272 – 3137)               | na        |
| <b>dsDNA (ng/mL)</b><br>(median, IQR)          | 13.8<br>(11.01-18.03)                  | 298.5<br>(76.5 - 517.9)        | 483.1<br>(252.1-766.5)        | 77.1<br>(30.7-138.3)          | 42.4<br>(41.9-51.7) | 392.3<br>(19.2-580.4)         | 63.7<br>(25.4-96.8)        | 742.3<br>(436.5-808.5)            | <0.0001   |
| <b>Dnase I (ng/mL)</b><br>(median, IQR)        | 8.1<br>(4.5-10.3)                      | 23.0<br>(12.5-37.1)            | 88.0<br>(31.0-204.7)          | 29.1<br>(23.1-41.2)           | 42.4<br>(41.9-51.7) | 15.6<br>(14.4-61.8)           | 15.8<br>(5.6-26.8)         | 28.7<br>(14.4-51.0)               | 0.007     |
| <b>BALF neutrophils (%)*</b>                   | 1.0<br>(1.0-2.3)                       | 68<br>(30.0-80.0)              | 4.0<br>(3.0-8.0)              | 9.0<br>(1.8-30.0)             | 4.0 (n=1)           | 30.0<br>(4.0-90.0)            | 54.0<br>(4.0-88.0)         | 85.0<br>(52.5-93.8)               | <0.0001   |
| <b>dsDNA plasma (ng/mL)</b><br>(median, IQR)   | 75.8<br>(60.7-99.1)<br>n=6             | 119.4<br>(82.7-210.8)<br>n=11  | 231.1<br>(119.9-265.8)<br>n=3 | 257.3<br>(17.3-497.3)<br>n=2  | Not available       | 444.6<br>(205.8-669.8)<br>n=5 | Not available              | 344.7<br>n=1                      | 0.2       |
| <b>Dnase I plasma (ng/mL)</b><br>(median, IQR) | 372.2<br>(248.6-527.2)<br>n=6          | 351.6<br>(207.2-492.3)<br>n=11 | 1110<br>(419.4-2522)<br>n=3   | 555.2<br>(406.8-703.6)<br>n=2 | Not available       | 443.3<br>(322.2-563.9)<br>n=5 | Not available              | 129<br>(n=1)                      | 0.11      |
| <b>ANC^</b><br>(median, IQR)                   | 1440<br>(670-2960)<br>n=11             | 3370<br>(1090-6710)<br>n=26    | 1920<br>(830-8220)<br>n=3     | 3315<br>(1333-6142)<br>n=6    | 30<br>n=1           | 3420<br>(2360-7095)<br>n=9    | 6395<br>(2598-8408)<br>n=4 | 4650<br>(2490-7915)<br>n=10       | 0.15      |

Only plasma samples collected within 4 days of BALF sample were analyzed (n=33).

\*Neutrophils in BALF reported as % of all inflammatory cells

\*\*p-value for trend. Individual comparisons in Figures 1 and 2.

\*\*\*Samples that were positive for infection in pre-transplant/screening bronchoscopy, were categorized as infection

^ ANC: absolute neutrophil count at the time of pulmonary event

Only plasma samples collected within 4 days of BALF sample were analyzed (n=33).

**Supplemental Table 2:** Correlations between BALF NETs, DNase I with BALF neutrophils and plasma (Spearman correlation).

A.

| Biomarker 1       | Biomarker 2      | r     | p-value |
|-------------------|------------------|-------|---------|
| BALF neutrophil % | BALF DNase I     | 0.39  | 0.0001  |
| BALF dsDNA        | Plasma dsDNA*    | 0.033 | 0.9     |
| BALF DNase I      | Plasma DNase I * | 0.15  | 0.23    |
| BALF dsDNA        | ANC              | 0.3   | 0.01    |
| BALF neutrophil % | ANC              | 0.4   | 0.005   |

\*Only plasma samples collected within 4 days of BALF sample were analyzed (n=33).

**Supplemental Figure 1: A.** BALF MCP-1 levels pre-transplant compared to post-transplant (median, IQR). **B.** BALF MCP-1 levels at the time of bronchoscopy per (p-values were generated in comparison to pre-tx/routine group using Mann Whitney U test and adjusted for multiplicity using a Bonferroni correction). (4000 pg/mL is upper limit of detection)

