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The RNFT2/IL3Rα Axis Regulates IL3 Signaling and Innate Immunity

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One Sentence Summary: The E3 ubiquitin ligase RNFT2 limits inflammation by targeting the cytokine receptor IL3Ra for degradation.

ABSTRACT
Interleukin-3 (IL3) receptor α (IL3Rα) is the alpha subunit of the ligand-specific IL3 receptor and initiates intracellular signaling in response to IL3. IL3 amplifies pro-inflammatory signaling and cytokine storm in murine sepsis models. Here we found that RNFT2 (RING finger transmembrane-domain containing protein 2, also TMEM118), a previously uncharacterized RING finger ubiquitin E3 ligase, negatively regulated IL3-dependent cellular responses through IL3Rα ubiquitination and degradation in the proteasome. In vitro, IL3 stimulation promoted IL3Rα proteasomal degradation dependent on RNFT2, and we identified IL3Rα Lysine 357 as a ubiquitin acceptor site. We determined that LPS-priming reduces RNFT2 abundance, extends IL3Rα half-life, and sensitizes cells to the effects of IL3, acting synergistically to increase pro-inflammatory signaling. In vivo, IL3 synergized with LPS to exacerbate lung inflammation in LPS and *Pseudomonas aeruginosa*-challenged mice; conversely, IL3 neutralization reduced LPS-induce lung injury. Further, RNFT2 over-expression reduced lung inflammation and injury, whereas *Rnft2* knockdown exacerbated inflammatory responses in LPS-induced murine lung injury. Lastly, we examined RNFT2 and IL3Rα in human lung explants from patients with Cystic Fibrosis, and also showed that IL3 is elevated in mechanically-ventilated critically ill humans at risk for Acute Respiratory Distress Syndrome (ARDS). These results identify RNFT2 as a negative regulator of IL3Rα, and show a potential role for the RNFT2/IL3Rα/IL3 axis in regulating innate immune responses in the lung.
INTRODUCTION

Interleukin-3 (IL3), a cytokine that regulates hematopoiesis (1), also amplifies cytokine storm in murine sepsis(2), and in septic humans elevated circulating IL3 is associated with mortality(2). The downstream effects of IL3 are mediated by binding to its receptor, a complex that contains an alpha subunit (IL3Rα or CD123) and a beta subunit (IL3Rβ or CSF2RB) (3). IL3Rα neutralizing antibodies are protective in murine sepsis (2, 4) and IL3 predicts responsiveness to corticosteroid therapy in septic humans (5). Thus, the IL3/IL3Rα axis may be a therapeutic target in sepsis and other acute inflammatory disorders. Here we show a previously uncharacterized mechanism regulating IL3Rα abundance through the ubiquitin/proteasome system (UPS), directed by the E3 ligase RING finger transmembrane-domain containing protein 2 (RNFT2).

IL3-initiated intracellular signaling is dependent on receptor subunits IL3Rα and IL3Rβ (1, 6). The beta subunit IL3Rβ, also known as the beta-common receptor (βc), is a shared receptor subunit that detects several colony-stimulating factor cytokines, including IL3, GM-CSF, and IL-5 (1, 3, 6). Depending on the stimulus (IL3, GM-CSF, or IL-5) and the cell type, several intracellular signaling pathways can be activated. The most well characterized signaling mechanisms include activation of the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) pathway and the PI3 kinase pathway(7). Further, activation of the NF-κB pathway through TRAF6 recruitment to the beta subunit has been described(8). The consequences of these signaling pathways is diverse and context specific, as βc cytokines have been implicated in several inflammatory diseases, including allergic asthma, inflammatory bowel disease, multiple sclerosis, and sepsis, among others (9). In murine sepsis, IL3 potentiates cytokine storm, a phenomenon associated with end-organ damage and mortality, and blockade of the IL3 receptor subunit IL3Rα protects against the deleterious effects of IL3(2).

Ubiquitin (Ub) conjugation is a post-translational modification of substrate proteins that involves at least three enzymes, including a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2) and a Ub ligase (E3). The E1 activates the C-terminus of ubiquitin by using ATP, which directs ubiquitin to E2. The E3 ligases transfer ubiquitin to substrate proteins, either from a thioester linkage on the E3 received from the E2, or by serving as adaptors that bring the substrate in proximity to the E2 for ubiquitin transfer (10, 11). Three classes of ubiquitin E3 ligases contribute to protein ubiquitination: RING E3 ligase, HECT E3 ligase and U-BOX E3 ligase (11, 12). The RING E3 ligases contain the most members, and these ligases harbor a unique RING finger domain.
that consists of two zinc finger type domains(13–15). Several E3 ligases have been shown to regulate innate immunity and inflammation in lung injury(16–21), and here we characterize the E3 ligase RNFT2 as negative regulator of IL3Rα.

In this study, we show the IL3 receptor subunit IL3Rα is targeted for ubiquitination and degradation by the E3 ligase RNFT2 (RING finger transmembrane-domain containing protein 2, also TMEM118). We identify IL3Rα Lys357 as a critical residue regulating IL3Rα stability. In vitro, IL3 promotes RNFT2-dependent IL3Rα proteasomal degradation, an effect mitigated by LPS-priming. Using models of LPS and Pseudomonas aeruginosa (PA) murine lung injury in vivo, we show that while exogenous IL3 administration amplifies inflammation and organ damage, while IL3 neutralization is protective. Further, in LPS murine lung injury models, RNFT2 over-expression conferred protection from the deleterious effects of IL3, while Rnft2 knockdown augmented IL3-dependent effects and lung injury severity. Lastly, in humans we show IL3 is elevated in subjects with Acute Respiratory Distress Syndrome (ARDS), and that IL3Rα and RNFT2 abundance are inversely correlated in lung homogenates from lung explants in cystic fibrosis patients. These data show RNFT2 is a previously unrecognized regulator of IL3-dependent cellular effects by controlling IL3Rα stability.

RESULTS

IL3Rα is degraded in the proteasome in response to IL3. We sought out to determine IL3Rα protein stability. We confirmed IL3Rα expression in MLE-12 (MLE) cells and RAW cells (Figure 1A and S1A). IL3Rα transcript was also present in RNA-Seq experiments from MLE cells (GEO accession GSE94680)(22). MLE cells were treated with cycloheximide (CHX) and IL3Rα half-life was observed to be ~ 6h; additionally, we found CHX-induced IL3Rα degradation was prevented by the proteasome inhibitor MG132, but not by the lysosomal inhibitor leupeptin (Figure 1A). We observed a similar half-life and preservation with MG132 treatment in RAW cells (Figure S1A). As IL3Rα protein is protected by proteasomal blockage, we hypothesized that IL3Rα degradation is regulated by the ubiquitin proteasome system. Indeed, expression of ubiquitin in MLE cells led to a dose-dependent decrease in IL3Rα protein (Figure S1B). Further, IL3Rα protein is retained following co-expression of ubiquitin and MG132 treatment (Figure 1B), an effect not seen with leupeptin treatment.

We next characterized the ubiquitination linkage type, as substrate ubiquitination controls
several diverse cellular functions (23). We performed UbiCREST analysis, in which HIS-tagged IL3Rα was expressed in MLE cells prior to MG132 treatment, HIS-pulldown, and in vitro exposure to de-ubiquitinases (DuB) with differential activity to specific ubiquitin chain linkages (24). We observed digestion of IL3Rα polyubiquitination by DuBs with affinity for K48-, K27-, K29-, K33-, K11-, and K63-type linkages (Figure S1C). K48-linked polyubiquitination is a common linkage associated with proteasomal degradation (23, 25). As an orthogonal approach, we co-expressed IL3Rα and ubiquitin constructs with specific K→R mutations, prior to MG132 treatment and IL3Rα pull-down, followed by immunoblotting. We observed that the expression of several mutants, especially K48R and K63R mutants, resulted in reduced poly-ubiquitinated-IL3Rα compared to WT-Ubiquitin (Figure S1D). Taken together, these results suggest IL3Rα ubiquitination is a signal for degradation. Ubiquitin is conjugated to substrate proteins at target lysine (K) residues, and we generated IL3Rα lysine-to-arginine (K→R) mutants to identify putative ubiquitin acceptor sites, as arginine (R) is unable to accept ubiquitin. We found the IL3Rα K357R mutant protein was resistant to degradation in CHX chase experiments (Figure 1C). Thus, K357 is a candidate ubiquitin acceptor site for IL3Rα. These experiments suggest that IL3Rα protein is regulated through ubiquitination leading to proteasomal degradation.

We examined whether IL3 stimulation would promote proteasomal degradation of IL3Rα and affect its downstream signaling. IL3Rα abundance decreased in a time- and dose-dependent manner following IL3 stimulation (Figure 1D), an effect that was mitigated by pre-treatment with MG132, but not leupeptin (Figure 1E). Additionally, IL3 stimulation reduced amounts of ectopic IL3Rα WT, but not IL3Rα K357R, in MLE cells (Figure 1F). These results suggest that IL3Rα undergoes ligand-induced degradation in the proteasome. Lastly, we examined if IL3Rα affected IL3 dependent downstream effects. TRAF6-dependent NF-κB activation has been shown as a cellular response to IL3 (8) and we observed that IL3 stimulation of MLE cells increased TRAF6 abundance as well as RelB and phospho-P100, proteins involved in the NF-κB signaling cascade (Figure 1G). Further, over-expression of IL3Rα augmented these effects (Figure 1G).

**RING Finger E3 ligase RNFT2 regulates IL3Rα stability and signaling.** We next investigated the mechanism involved in IL3Rα protein ubiquitination and degradation. We over-expressed E3 ligases in MLE cells (18, 26–28) and observed that the uncharacterized E3 ligase RNFT2 decreased IL3Rα protein amounts (Figure 2A), without affecting its mRNA expression (Figure
Expression of RNFT2 led to a dose-dependent decrease of IL3Rα protein, but not that of IL3Rβ protein (Figure 2B). We observed that RNFT2 protein associated with IL3Rα in MLE cells by RNFT2 pull-down (Figure 2C). Thus, RNFT2 is a candidate E3 ligase targeting IL3Rα as a substrate for ubiquitination and degradation.

To confirm that RNFT2 functioned as an E3 ligase to ubiquitinate IL3Rα, we performed an in vitro ubiquitination assay. RNFT2 protein in addition to the full complement of ubiquitination machinery was sufficient to ubiquitinate IL3Rα in vitro (Figure 2D). As a complementary approach, we showed that co-expression of RNFT2 with HA-Ubiquitin and IL3Rα-HIS-V5 led to degradation of IL3Rα protein and increased the HA-ubiquitin signal upon IL3Rα-HIS pulldown as compared to control (Figure 2E). As a putative RING Finger E3 ligase, RNFT2 functions through its RING domain (Figure S2B). Mutation of critical residues within this region led to preservation of IL3Rα protein relative to WT (Figure S2C). To further confirm RNFT2 activity as an authentic ubiquitin E3 ligase, we performed in vitro binding assays and observed that RNFT2 indeed bound to several ubiquitin E2 enzymes (Figure 2F). We also confirmed the association of RNFT2 with endogenous UBE2D protein through cell-based pull-down assays (Figure 2G). Lastly, to demonstrate the specificity of RNFT2 to target IL3-Rα, we co-over-expressed RNFT2 with WT or K357R IL3-Rα. RNFT2 over-expression reduced amounts of IL3Rα WT, but not IL3Rα K357R (Figure 2H). Hence, we confirmed that RNFT2 is an authentic E3 ligase that targets IL3Rα as a substrate.

Next, we investigated whether RNFT2 affected IL3Rα abundance and signaling in response to IL3. RNFT2 overexpression significantly enhanced rIL3-induced IL3Rα degradation (Figure 3A), whereas Rnft2 knockdown markedly preserved IL3Rα protein with IL3 stimulation (Figure 3B). We found that ectopic expression of RNFT2 decreased TRAF6 protein amount in rIL3-stimulated MLE cells (Figure 3C), whereas Rnft2 knockdown significantly increased TRAF6 protein abundance (Figure 3D).

IL3 augments pro-inflammatory cellular responses to LPS through IL3Rα and RNFT2. IL3 exacerbates the pro-inflammatory effects of LPS (2) We hypothesized that this effect could be partially mediated through LPS-dependent modulation of IL3Rα stability. LPS treatment in MLE cells led to an increase in IL3Rα protein abundance and a concomitant decrease in RNFT2 protein levels (Figure 4A, B). While RNFT2 protein levels did not differ over an 8-hour CHX chase with
or without LPS co-treatment, we observed that LPS treatment decreased \textit{Rnft2} gene transcription (Figure S2D, E). The LPS-mediated effect on IL3R\textsubscript{α} protein levels was specific, as IL3R\textsubscript{β} protein levels were not affected by LPS (Figure 4C). Further, the increase in IL3R\textsubscript{α} protein abundance was independent of changes in \textit{IL3Rα} gene transcription (Figure 4D). We also found that in CHX chase experiments, LPS pre-treatment extended IL3R\textsubscript{α} half-life (Figure 4E). Co-treatment of LPS with IL3 increased supernatant cytokine levels of IL-6 and CXCL-1 compared to LPS treatment alone, an effect further exacerbated with IL3R\textsubscript{α} over-expression (Figure 4F, G).

We then examined the effect of RNFT2 over-expression or knockdown on cytokine secretion in MLE cells in response to LPS/IL3. RNFT2 over-expression mitigated the effect of LPS/IL3 co-treatment on CXCL-1 secretion (Figure 4H), while \textit{Rnft2} knockdown exacerbated this effect (Figure 4I). Lastly, we co-over-expressed WT or K357R IL3R\textsubscript{α} with an empty vector or RNFT2 and stimulated MLE cells with LPS/IL3. RNFT2 over-expression mitigated TRAF6 abundance as well as IL-6 and CXCL-1 cytokine secretion in cells co-overexpressed with WT IL3R\textsubscript{α}; however, RNFT2 over-expression had no effect on TRAF6 abundance, or IL-6 and CXCL1 cytokine secretion in cells co-overexpressed with K357R IL3R\textsubscript{α} (Figure S3A-C). Thus, the degradation-resistant K357R IL3R\textsubscript{α} mutant was not affected by RNFT2 over-expression. Taken together, these data suggest that LPS sensitizes cells to IL3 through stabilization of IL3R\textsubscript{α}.

**IL3 augments LPS-induced murine lung injury.** To determine whether IL3 contributed to the pathology of lung injury we used a clinically relevant model for pneumonia and severe lung injury initiated by intratracheal administration of LPS. After we treated mice with vehicle (PBS), LPS, recombinant IL3 (rIL3), or their combination intratracheally, we determined lung histology and protein concentration in Bronchoalveolar lavage fluid (BALF) were assayed 18 h later. LPS increased BALF protein amounts, and the abundance of the inflammatory cytokines IL-6, TNF-\textalpha, & IL-1\beta, and the DAMP HMGB1 in BALF. In contrast, IL3 treatment alone had only a modest effect on the same parameters. However, co-treatment of IL3 with LPS dramatically increased the amount of BALF protein, and abundance of the inflammatory cytokines IL-6, TNF-\textalpha, & IL-1\beta, and HMGB1 (Figure S4A-E) compared to LPS alone. BALF cell counts also displayed a similar pattern, with IL3/LPS co-treatment increasing BALF cell counts most dramatically among treatment groups (Figure S4F). Additionally, we observed increased numbers of BALF neutrophils and lymphocytes, but not macrophages, in mice co-treated with IL3 and LPS compared to PBS,
LPS, or IL3 alone (Figure S4G-I). Lung histopathology was consistent in that IL3/LPS co-treatment caused the most severe lung injury compared to PBS, LPS, or IL3 alone (Figure S4J). Taken together, these results suggest that IL3 amplifies LPS-induced lung injury.

**IL3 neutralization reduces inflammation in LPS-induced murine lung injury.** In separate experiments, we co-treated mice with LPS and either IgG (control) or a neutralizing antibody against IL3. We observed that BALF protein, and inflammatory cytokines IL-6, TNF-α, & IL-1β, and HMGB1 abundance were all significantly reduced in mice treated with anti-IL3 compared to IgG-treated control mice (Figure S5 A-E). Further, blocking-IL3 treatment reduced BALF cell counts and neutrophils but had no effect on macrophage or lymphocyte counts (Figure S5F-I). Blocking IL3 antibody co-treatment reduced the severity of lung injury in histopathological sections (Figure S5J). Thus, IL3 may drive inflammation in LPS-induced lung injury.

**The RNFT2/IL3Rα/IL3 axis regulates lung inflammation in vivo and in vitro.** We next examined the role of RNFT2 during acute lung injury. RNFT2 is an uncharacterized E3 ligase. We first performed IHC on mouse lung sections and confirmed RNFT2 was expressed in the mouse lung (Figure S6A). We then measured RNFT2, IL3Rα, and IL3Rβ protein amounts in lung homogenates after intratracheal LPS and observed that RNFT2 abundance decreased, IL3Rα abundance increased, and IL3Rβ amounts were unchanged compared to control mice (Figure 5A, B). We also observed increased IL3 in the BALF of LPS-treated mice compared to controls (Figure S6B) To validate these observations in another infectious acute lung injury model, we treated mice with *Pseudomonas aeruginosa* (PA) and measured abundance of RNFT2, IL3Rα, and IL3Rβ and observed a similar pattern of protein signal, and of IL3 signal in murine BALF (Figure S6C-G).

To determine the role of RNFT2 in LPS/IL3-induced lung injury, we infected mice intratracheally with empty lentivirus or lentivirus encoding *Rnft2*, and subsequently challenged them with LPS with or without rIL3. *Rnft2* gene transfer significantly reduced lung inflammation and injury caused by LPS and rIL3 co-treatment as shown by decreased BALF cell counts, protein concentrations, cytokine release, and lung infiltrates (Figure 5C-G, K). Specifically, the BALF leukocyte differential revealed that the total decrease in inflammatory cells was mostly from neutrophils and lymphocytes, but not macrophages (Figure S7A-C).

Next, we administered lentivirus intratracheally encoding control shRNA or *Rnft2* shRNA and
then exposed to LPS with or without rIL3. Rnft2 knockdown significantly enhanced lung inflammation and injury caused by LPS and rIL3 treatment as shown by increased BALF cell counts, protein concentrations, cytokines release, and lung infiltrates (Figure 5H-J, L). Specifically, the differential cell counts of BALF revealed that the total increase in inflammatory cells was mostly from neutrophils and lymphocytes, but not macrophages (Figure S7D-F). These results suggest that RNFT2 modulates lung inflammation in vivo.

**The IL3/IL3Rα/RNFT2 axis is relevant in human inflammatory lung disease.** To determine protein expression patterns of IL3Rα and RNFT2 in clinically relevant human disease, we assayed parenchymal explant lung samples from human cystic-fibrosis patients colonized with PA by immunoblot for RNFT2 and IL3Rα protein amounts, and we quantified their relative expression normalized to Tubulin. We observed a significant negative correlation between RNFT2 and IL3Rα protein signal (Figure 6A) (Pearson r²=0.278). These studies suggest that the RNFT2/IL3Rα pathway may be relevant in response to PA infection in patients, where decreased amounts of RNFT2 result in reciprocally increased IL3Rα abundance.

Circulating IL3 may be a potentiator of cytokine storm, and in a cohort of 60 patients with septic shock, high IL3 (>89.4 pg/mL) amount were associated with mortality. In this cohort, there was no significant difference in circulating IL3 protein abundance at Day 0 vs. Day 1 or Day 2 of following sepsis diagnosis (2). However, in a separate cohort, IL3 was significantly elevated in septic patients relative to healthy controls (29). We sought out to determine if circulating IL3 differed in a more homogenous population at risk for ARDS (acute respiratory distress syndrome), as a subset of patients at risk or with ARDS have excessive inflammation and cytokine storm in the lung (30, 31). We assayed IL3 in plasma from mechanically-ventilated subjects at risk for ARDS vs. mechanically-ventilated subjects without lung injury (Table 1 and Supplemental Table 1). Subjects at-risk for ARDS had increased plasma IL3 compared to subjects without lung injury (Figure 6B). Further, IL3 levels were associated with other plasma pro-inflammatory cytokines in this cohort, including TNFα and IL1β (Figure 6C-D). We also measured RNFT2 gene expression through RT-qPCR from white blood cell pellets from the same subjects, but found no significant correlations to the same pro-inflammatory cytokines (Figure 6E-F). Thus, high circulating IL3 may be an additional biomarker to identify subjects at risk for ARDS with excessive lung inflammation. Further studies with increased power are warranted to determine if IL3 could be a new biomarker.
in patients with or at risk for ARDS.

In summary, our studies demonstrate IL3Rα stability is regulated through the ubiquitin-proteasome system, dependent on the previously uncharacterized E3 ligase RNFT2. These findings may have implications for pulmonary inflammatory disorders including acute lung injury. We characterize RNFT2 as an authentic E3 ligase that directs IL3Rα ubiquitination and degradation and identify Lysine 357 on IL3Rα as a critical residue regulating its stability, as K357R IL3Rα mutants are resistant to RNFT2-directed degradation. In vivo, we show 1.) IL3 exacerbates LPS-induced lung injury, 2.) IL3 neutralization reduced inflammation in LPS-induced lung injury, 3.) RNFT2 over-expression is protective in LPS/IL3-induced lung injury and mitigates the effects of IL3, and 4.) Rnft2 knock-down exacerbates LPS/IL3 induced lung injury, and exacerbates the effects of IL3.

DISCUSSION

IL3 has recently been implicated in the pathogenesis of several inflammatory disorders, including collagen-induced arthritis (32), autoimmune encephalitis (33), and lupus nephritis (34). In some of these models, IL3 blockade reduced disease severity scores and was associated with decreased systemic inflammatory markers, whereas administration of IL3 exacerbated these effects(33, 34). Our findings are in line with these studies, as we show that IL3 blockade reduces lung injury and inflammatory markers in a murine model of severe lung injury, and that addition of IL3 exacerbates organ damage. Our results are also consistent with those of Weber, et al, who demonstrated that IL3 alone is not sufficient to induce a systemic inflammatory response, but rather serves to “amplify” signaling initiated by an initial insult (CLP, or intra-tracheal LPS) (2). Here we extensively studied the role of the IL3/IL3Rα axis in lung innate immunity and inflammation. We found IL3 amounts were increased in BALF of mice subjected to LPS or Pseudomonas (PA) treatment, and rIL3 co-treatment with LPS drastically enhanced pro-inflammatory cytokine release and immune cell activation in murine lungs. We observed IL3 treatment synergizes with bacterial PAMP-exposure to strongly activate downstream signaling through NF-κB activation, leading to pro-inflammatory cytokine release. Our model is consistent with the ‘second-hit’ theory or multi-causal effect mentioned in previous studies (35, 36).

IL3 classically signals through the JAK/STAT pathway (9), but has also been linked to NF-κB through tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) (8). TRAF6 is one
of the six well-characterized TRAF proteins, which are critical contributors to inflammatory, innate, and adaptive immune responses (28, 37, 38). Specifically, TRAF6 is a key mediator of TLR signaling and downstream NF-κB signaling (39–41). We found TRAF6 increased in MLE cells treated with rIL3, suggesting TRAF6 is a candidate contributor to the synergy of IL3 and NF-κB activation. We also observed that ectopic expression of the IL3 receptor (IL3Rα) significantly increased TRAF6 amount and enhanced cytokine release upon IL3/LPS co-treatment. These studies suggest that IL3 along with its receptor IL3Rα synergize with bacterial PAMP exposure through downstream NF-κB signaling.

In this study, we found that IL3Rα protein abundance is regulated post-translationally through ubiquitination. Protein ubiquitination is a universal cellular mechanism controlling protein amount and innate immunity (20, 42–45). Recently, we have shown that several E3 ligases regulate substrate ubiquitination in pulmonary inflammatory conditions (17, 18, 21). During systemic insults induced by infection, alterations of E3 ligase activity or targeting can affect substrate protein abundance and their downstream effector functions. We showed that intratracheal treatment of LPS or PA in vivo increases substrate IL3Rα protein amount, and that LPS treatment of MLE cells increases IL3Rα. Lung tissue from PA-treated mice showed decreased abundance of the putative E3 ligase RNFT2, with a correlative increase in IL3Rα protein, suggesting the exogenous insult manipulated RNFT2 protein amount, and thus the protein stability of its substrate IL3Rα. We characterized RNFT2 as an authentic E3 ligase by showing its interaction with the E2 enzyme UBE2D in vitro. We further found that RNFT2 regulated the rIL3-induced increase in TRAF6 protein and affected CXCL-1 secretion in MLE cells. Specifically, ectopic expression of RNFT2 in MLE cells significantly suppressed co-treatment (LPS with rIL3) induced cytokine release, whereas Rnft2 knockdown enhanced CXCL-1 secretion. RNFT2 gene transfer and knockdown in vivo also has similar phenotype in cytokine release and immune cell activation such as neutrophils in lung. Our data imply that RNFT2 functions as an inflammatory suppressor protein that controls IL3 signaling in innate immunity, and the RNFT2/IL3Rα axis may be important in the lung innate immune response chain.

This study also provided two additional human translational links that suggest the IL3/IL3Rα signaling axis may play a role in human inflammatory lung diseases. We show that plasma IL3 protein amounts are increased in humans at risk for ARDS compared to mechanically ventilated subjects without lung injury. These findings warrant examination in larger lung injury cohorts and
suggest that IL3 may be a previously unrecognized cytokine associated with excessive inflammation. We also examined protein abundance of RNFT2 and IL3Rα in explanted lung tissue from patients with chronic pseudomonas infection. We discovered there was an inverse correlation between RNFT2 and IL3Rα protein amount, suggesting that our newly described mechanism of RNFT2-dependent IL3Rα degradation may be operant in humans with chronic infection. Further studies are warranted to delineate the role of RNFT2 in these inflammatory disorders.

There are limitations to these studies. While our in vivo work clearly identifies a role for IL3 in LPS and PA murine acute lung injury, we do not identify the cell type responsible for IL3 secretion. In murine sepsis models, IRA-B cells are thought to be responsible for IL3 secretion (2, 46). Further, T cells and B cells are classically regarded as the main IL3 producers in other models of disease (7, 9, 32, 33). Whether IRA-B cells are also responsible for IL3 secretion in acute lung injury models is an active area of investigation. Further, whether these cells express RNFT2 is also unknown. Recent single cell RNA sequencing studies on murine airway cells showed no detection or extremely low levels of IL3 gene expression, which was not significantly altered among different airway cell types (47–49), suggesting that lung cells are not a primary producer of IL3. Additionally, while we show in vitro that IL3Rα abundance increases and RNFT2 abundance decreases in LPS-stimulated MLE12 cells, the changes in RNFT2 and IL3Rα abundance in lung homogenates after in vivo during lung injury could also be explained by differences in infiltrating immune cells between control and LPS/PA-treated mice. Thus, while our in vitro work provides mechanistic insight into RNFT2/IL3Rα, additional work remains to further characterize this pathway in vivo.

We use the MLE12 cell line to characterize IL3Rα stability and test the role of RNFT2 in modulating IL3Rα-dependent downstream effects. MLE12 cells are an immortalized cell line that closely resembles, but does not fully mirror, Type II alveolar epithelial cells. We have identified several E3 ligase/substrate interactions relevant in pulmonary inflammatory conditions using the MLE-12 cells (28, 50–52). Future studies will focus on examining the RNFT2/IL3Rα expression in other pulmonary cell types, including primary human cells. We chose to examine NF-κB signaling (TRAF6 abundance, IL-6 secretion, CXLC-1 secretion) because our laboratory has previously shown role for TRAFs in experimental lung injury (28), and IL3 signaling has been linked to TRAF6 recruitment(8). However, whether RNFT2 also has similar effects on IL3Rα-dependent signaling in immune cells is unknown.
Lastly, our in vitro findings provide a framework to interrogate the role of RNFT2 in vivo in pathological disease states where IL3 is implicated. Related to our studies, it will be critical to determine which cell types are responsible for mediating the effects we observed after intratracheal lentiviral RNFT2 over-expression or knockdown. More broadly, these findings could have implications in other disease states where IL3Rα expression is altered. For example, IL3Rα is over-expressed in several hematologic malignancies and is the basis for novel therapies including chimeric antigen receptor (CAR) T cell therapies (53, 54). The role of RNFT2 in these conditions is unknown, and one could postulate that reduced RNFT2 expression or function could be partially responsible for aberrant IL3Rα expression. Further, pharmacological targeting of RNFT2 with a small molecule inhibitor could potentially enhance IL3Rα expression.

In conclusion, we showed that IL3 synergizes with LPS to exacerbate lung inflammatory injury, resulting in increased NF-κB activation and lung injury. This synergy proceeds through the IL3Rα subunit, whose protein stability is weakened by IL3 treatment but preserved by LPS exposure. IL3Rα protein is processed by the ubiquitin proteasome system, and the previously uncharacterized RING E3 Ligase, RNFT2, targets IL3Rα for proteasomal degradation by ubiquitinating it at lysine 357. The RNFT2/IL3Rα axis controls pro-inflammatory cytokine and immune cell release in murine lung inflammation models. Taken together, this study characterized a regulatory mechanism involving RNFT2-targeted degradation of the IL3 cytokine receptor IL3α.

MATERIALS AND METHODS

Reagents

Anti-IL3Rα antibodies (MBS2542745; sc-74522) were obtained from MYBioSource and Santa Cruz Biotechnology. Anti-IL3Rβ (MBS2534790) antibody was from MYBioSource. Monoclonal anti-HA-Tag (clone 6E2) (Cat# 2367S RRID:AB_10691311), Anti-TRAF6 (Cat# 8028, RRID:AB_10858223), Anti-RelB (Cat# 10544, RRID:AB_2797727), and Anti-Phos-P100 (Cat# 4810, RRID:AB_659925) were from Cell Signaling Technology. Anti-V5 antibody (R960, RRID:AB_159298) was from Invitrogen. Anti-Actin antibody (A5441, RRID:AB_476744) and leupeptin (L2884) were from Sigma Aldrich. Goat anti-Rabbit IgG-HRP (Cat# 170-6515 RRID:AB_11125142), Goat anti-Mouse IgG-HRP (Cat# 170-6516 RRID:AB_11125547), and CFX96 Touch Real-Time qPCR (1855196) were from BioRad. MG-132 (F1100) was from
UBPBio. Recombinant Murine IL3 was from PeproTech. Murine CXCL1 (DY453) and Murine TNF-a (DY410) ELISA kits were from R&D Systems. Murine IL-1β (88–7261) and murine IL-6 (88–7064, RRID: AB_2574986) ELISA kits were from eBioscience. SYBR Select Master Mix (4472918) was from Life Technologies. QuikChange II XL Site-Directed Mutagenesis Kit (200522) was from Agilent Technologies. High capacity RNA-to-cDNA kits (4387406) were from Applied Biosystems.

**Cell Culture**

Murine Lung Epithelial 12 cells (MLE) (CRL-2110) were obtained from ATCC and cultured in Dulbecco’s Modified Eagle Medium-F12 (Gibco) with 10% fetal bovine serum (DMEM-F12-10). For RNFT2 overexpression in MLE cells, electroporation was used, and 24 or 48hr later, cells were treated with stimulators. For RNFT2 knockdown, scramble siRNA and RNFT2 siRNA were used to transfect MLE cells for 48hr by electroporation. Cell lysates were collected in Lysis Buffer (150 mM NaCl, 50 mM Tris, 1.0 mM EDTA, 2 mM dithiothreitol, 0.025% sodium azide, and 1 mM phenylmethylsulfonyl fluoride), and prepared by brief sonication at 4°C. Additionally, MLE cells were treated with cycloheximide (100μg/ml) for half-life studies. RAW264.7 cells were from ATCC (TIB-71) and cultured in DMEM with 10% fetal bovine serum. THP-1 cells were from ATCC (TIB-202) PBMCs and THP-1 cells were cultured in RPMI Medium 1640 (Gibco) with 10% fetal bovine serum. Pseudomonas aeruginosa (PA103) was from ATCC (29260).

**RT–qPCR, cloning, and mutagenesis**

Total RNA was isolated and reverse transcription was performed followed by real-time quantitative PCR with SYBR Green qPCR mixture as described (18).

**In vitro ubiquitin conjugation assays**

The assay was performed in a volume of 20μl containing 50 mM Tris, pH 7.6, 5mM MgCl2, 0.6mM DTT, 2mM adenosine triphosphate (ATP), 10μM MG132, 100nM Ubiquitin activating enzyme, 0.5 μM UBE2D, 2μM ubiquitin, and 1μM ubiquitin aldehyde. TnT-coupled reticulocyte in vitro–synthesized proteins (tagless-RNFT2 and HIS-purified IL3Rα-V5) and reaction products were processed for V5 immunoblotting.
Plasmid Transfection
For protein overexpression in MLE-12 cells, cells were nucleofected using Nucleofector 2B (Amaxa), program T-13. For protein overexpression in HEK293T cells, XtremeGENE transfection reagents were used following the manufacturer’s protocol. Expression was confirmed by western blotting.

Recombinant DNA Constructs
Total RNA was isolated from untreated human PBMCs using RNeasy Mini Kit, and reverse transcribed to cDNA using High-Capacity RNA to cDNA kits. RNFT2 cDNA were PCR amplified, isolated, and sequence confirmed prior to PCR-cloning into pcDNA3.1D-V5-HIS vector. Deletion mutants were generated by PCR-cloning. Point mutants of RNFT2 and IL3Rα were generated using the QuikChange II XL kit, per the manufacturer’s protocol. pRK5-HA-Ubiquitin-WT was a gift from Ted Dawson (Addgene plasmid # 17608, (55)) (Johns Hopkins University School of Medicine, Baltimore, MD)

In vitro protein binding assays
RNFT2 protein was immunoprecipitated from 1 mg MLE cell lysate using 1:100 dilution of RNFT antibody (rabbit) for four hours at 25°C in IP buffer (50 mM Tris HCl pH 7.6, 150 mM NaCl, 0.25 % v/v Triton-X-100) and coupled to protein A/G agarose resin for an additional hour. V5-Ubiquitin E2 enzymes (20 μl) were in vitro synthesized using TnT translation kits for 90 minutes at 30°C. RNFT2-bound resin was then incubated with the in vitro synthesized proteins for 18 hours at 4°C. Following binding, resin was washed with IP buffer, and eluted by dilution in denaturing loading buffer, with a final 1X formulation of: 50 mM Tris HCl pH 6.8, 2% SDS, 10% Glycerol and 100 mM DTT and incubation at 88°C for five minutes prior to immunoblotting analysis.

Immunoprecipitation Assays
MLE cells were cultured and treated as indicated prior to collection. Cell pellets were lysed with IP buffer (0.25% Triton-X-100 in 1x PBS, pH 7.6, 0.025% sodium azide, and 1 mM phenylmethylsulfonyl fluoride) on ice. Lysates were prepared by brief sonication at 4°C. Insoluble cellular debris was precipitated through centrifugation at 15,000x rcf for 10 min at 4°C. Lysate supernatant was normalized for protein concentration. Supernatants were exposed to a 1:50
dilution of the indicated antibody for three hours at 25°C. Immunoprecipitated protein was captured with 10µL magnetic protein A/G resin for 1 hour prior to two rounds of washing with IP buffer (1 mL). Protein was eluted by dilution in denaturing loading buffer, with a final 1X formulation of: 50 mM Tris HCl pH 6.8, 2% SDS, 10% Glycerol and 100 mM DTT and incubation at 88°C for five minutes. Eluted samples were resolved by SDS-PAGE and subjected to immunoblotting.

**HIS-Pulldowns Assays**

MLE cells were cultured and treated as indicated prior to collection. Lysate was recovered in PD buffer (0.25% Triton-X-100 in 1x PBS pH 8.0, 30mM imidazole, and 1 mM phenylmethylsulfonyl fluoride) on ice, before brief sonication and sedimentation by centrifugation (15,000x rcf, 10min, 4°C). Lysate supernatants were subjected to HIS-pulldown by 10µL magnetic HisPur resin for 1 hour prior to two rounds of washing with PD buffer (1mL). Protein was eluted by dilution in denaturing loading buffer, with a final 1X formulation of: 50mM Tris HCl pH 6.8, 2% SDS, 10% Glycerol and 100mM DTT and boiling for 5 minutes. Eluted samples were resolved by SDS-PAGE and subjected to Immunoblotting.

**Animal studies**

C57BL/6J mice were purchased from the Jackson Laboratory. Animals were between 7-9 weeks of age and around 25g weight at time of experiment. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Models of endotoxin-induced lung injury (IT LPS) or Lung Injury by Live Bacteria (IT *Pseudomonas*) were employed in accordance with ATS guidelines on animal models of experimental lung injury(56). The principal objective in these models is the physiological assessment of lung injury, and studies were powered to detect a two-fold difference BAL protein amount, which is a well-established primary end point for animal models of ALI, and based on our prior published results using these models of lung injury (17, 18, 21, 27, 28, 50, 57). For intratracheal injection studies, C57BL/6J mice were anesthetized using a ketamine/xylazine mixture, and the larynx was well visualized under a fiber optic light source before endotracheal intubation with a 3/400 24-gauge plastic catheter. LPS with or without IL3 (rIL3) (LPS: 1.5mg/kg; rIL3: 6µg) were administered i.t. for 18h, after which animals were euthanized and assayed for BAL protein amounts by Lowry assay and
immunoblotting, cell count by TC20 automated cell counter, cytokine abundance by ELISA, and lung tissue H&E staining. In another study, LPS with or without IL3 neutralization antibody (Cell Signaling, Cat#D6C1) (LPS: 1.5mg/kg; α-IL3: 5µg) were administered i.t. for 18h, after which animals were euthanized and assayed for BAL protein amounts by immunoblotting, cell count, cytokine abundance by ELISA, and lung tissue H&E staining. For lentiviral study, 1x10^7 PFU of lentivirus encoding genes for RNFT2 or Rnft2 shRNA was treated i.t. for 144 hr before the administration of LPS with or without rIL3 (LPS: 1.5mg/kg; rIL3: 6µg in RNFT2 overexpression mice, 3µg in RNFT2 shRNA mice) for 18 hr, after which animals were euthanized and assayed for BAL protein amounts by immunoblotting, cell count, cytokine abundance by ELISA, and lung tissue H&E staining.

**Human Studies (Acute Respiratory Distress Syndrome (ARDS))**:

De-identified human plasma samples were obtained from the University of Pittsburgh Acute Lung Injury Biospecimen Repository (IRB number #PRO10110387). Informed consent was provided by all participants or their surrogates in accordance with the Declaration of Helsinki. Adults aged 18-90 admitted to the Medical ICU in the UPMC Presbyterian Hospital with acute respiratory failure requiring mechanical ventilation by endotracheal tube were enrolled as patients if the onset of acute respiratory failure was associated with established risk factors for developing acute lung injury (sepsis, pneumonia, aspiration, blood transfusion, pancreatitis, or trauma) or if intubation was performed for airway protection in the setting of non-pulmonary critical illness. Enrollment took place within 48 h of the initiation of mechanical ventilation. After enrollment, patients were retrospectively sub-classified by an expert clinical panel into categories: 1) ‘ARDS’, as defined by Berlin criteria and agreed upon by a minimum of three members of an expert clinical panel, 2) ‘at risk,’ as defined as Lung Injury Prediction Score (LIPS) score >4 and the presence of a clinically identifiable risk factor for ARDS, and 3) ‘ventilated control,’ as defined by intubation and mechanical ventilation for non-pulmonary critical illness without risk factor for ARDS. We performed a preliminary sample size calculation to detect a three-fold difference in two means (30 pg/mL vs. 90 pg/mL) between ventilated control and lung injured-patients with a power of 0.8, α of 0.05, an estimated standard deviation of 35, and a sampling ratio of 0.25 ventilated control/lung-injured patients (given there are fewer control patients enrolled in our study). We chose values of 30 and 90 pg/mL based on published literature showing an IL3 of >89.4 pg/mL is associated with
poor outcomes in sepsis (2), and estimated baseline amounts from manufacturer data and published literature. The predicted samples size was 4 “control” patients and 14 “lung-injured” patients. With this, \(n=4\) ‘ventilated control,’ \(n=16\) ‘at risk for ARDS,’ were randomly selected from the cohort for analysis.

**Human Studies (Cystic Fibrosis, CF):**
Following attainment of informed consent, CF lung tissue was obtained from excess pathological tissue following lung transplantation in accordance with a protocol approved by the University of Pittsburgh Investigational Review Board. 1cm\(^2\) sections of lung parenchyma were frozen and stored at -80 until use. ~100mg of lung tissue were homogenized in RIPA buffer followed by protein immunoblotting.

**Statistics** — Protein Signal densitometry was quantified by ImageJ. Comparisons of two groups were carried out with unpaired, two-tailed Student’s T-test. Comparisons of more than two groups were tested with One-ANOVA with Tukey or Dunnett’s post-hoc test for multiple comparisons as noted. Non-linear regression plots were tested with F-Test. All tests used \(p < 0.05\) as indicative of statistical significance. All statistical analyses were carried out using the Graph Pad Prism 8.0 program.

**Study Approval** — C57BL/6J mice were purchased from the Jackson Laboratory. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. For ARDS human studies, de-identified human plasma samples were obtained from the University of Pittsburgh Acute Lung Injury Biospecimen Repository (IRB number #PRO10110387). Informed consent was provided by all participants or their surrogates in accordance with the Declaration of Helsinki. Following attainment of informed consent, CF lung tissue was obtained from excess pathological tissue following lung transplantation in accordance with a protocol approved by the University of Pittsburgh Investigational Review Board.

**Author contributions:** B.B.C., Y.L., and M.J.J designed the study, analyzed the data; Y.T., T.L., J.E. and B.B.C. wrote the manuscript; Y.T., T.L., J.E., K.C.L, Y.C., and J.D.L. performed all in vitro experiments and animal experiments; J.E., M.M.M, Y.Z., I.D.P., J.F.M, and B.J.M. designed
and executed the human studies. B.B.C. directed the study

SUPPLEMENTARY MATERIALS

Figure S1. IL3Rα is ubiquitinated and assembles specific poly-ubiquitin linkage types.
Figure S2. RNFT2 is a RING Ubiquitin E3 ligase
Figure S3. RNFT2 effect on inflammation proceeds through IL3Rα
Figure S4. IL3 aggravates LPS-induced lung injury in vivo
Figure S5. IL3 blocking antibody attenuates LPS-induced lung injury.
Figure S6. Bacterial infection influences Rnft2:Il3ra protein levels
Figure S7. RNFT2 regulates neutrophils counts in lung inflammation and injury

References and Notes


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Competing interests: The authors declare that they have no conflicts of interest.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.
FIGURE LEGENDS

Figure 1: IL3Rα is degraded in the proteasome in response to IL3. (A) Immunoblot analysis of IL3Rα protein abundance in MLE cells treated with cycloheximide (CHX) with or without MG-132 or Leupeptin in a time-dependent manner. Data and means ± SEM of 3 independent experiments. (B) Immunoblotting of IL3Rα-V5 HIS pull-down (HIS PD) from MLE cells after co-expression of IL3Rα-V5 and HA-Ubiquitin, and treatment with MG132 or leupeptin. (C) Immunoblot analysis of MLE cells transfected with WT or lysine mutant (K357R) IL3Rα and treated with CHX. Data and means ± SEM of 3 independent experiments. (D) Immunoblotting of IL3Rα protein abundance from MLE cells treated with recombinant IL3 protein (rIL3) in a dose or time-dependent manner. (E) Immunoblot analysis of IL3Rα protein amount from MLE cells treated with rIL3 with or without MG-132 or Leupeptin in a time-dependent manner. Data and means ± SEM of 3 independent experiments. (F) Immunoblot analysis of IL3Rα-V5 protein amount from MLE transfected with WT or K357R mutant IL3Rα and treated with rIL3 treatment. Data and means ± SEM of 3 independent experiments. (G) Immunoblot analysis of MLE cells transfected with IL3Rα or empty plasmid, and then were challenged with or without rIL3. Data and means ± SEM of 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 by F-test (A, C, E, F), or by one-way ANOVA with Tukey’s post hoc test (G).

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**Figure 4. IL3 augments pro-inflammatory cellular responses to LPS through IL3R\(\alpha\) and RNFT2.** (A-C) Immunoblot analysis of IL3R\(\alpha\) (A), RNFT2 (B), and IL3R\(\beta\) (C) protein abundances in Murine lung epithelial (MLE) cells treated with LPS at the indicated times. Blots (top) are representative of 3 independent experiments. Quantified data (lower) are means ± SEM from all experiments (n=3). (D) RT-qPCR analysis of IL3R\(\alpha\) mRNA expression in Murine lung epithelial (MLE) cells treated with LPS at the indicated times. Quantified data are means ± SEM from all experiments (n=3). (E) Immunoblot of IL3R\(\alpha\) in MLE cells treated with cycloheximide (CHX) and control (Con) or LPS, as indicated. Blots (top) are representative of 3 independent experiments. Quantified data (lower) are means ± SEM from all experiments (n=3). (F and G) ELISA analysis of IL-6 and CXCL-1 in supernatants from MLE cells transfected with IL3R\(\alpha\) or empty plasmid, and challenged with LPS and rIL3, as indicated. Data are means ± SEM from all experiments. (H) ELISA analysis of CXCL-1 in MLE cells transfected with RNFT2 or empty plasmid before treatment with LPS with or without rIL3. Data and means ± SEM of 3 independent experiments. (I) ELISA analysis of CXCL-1 in MLE cells transfected with con-siRNA or RNFT2
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Figure 5. The RNFT2/IL3Rα axis regulates lung innate immunity and inflammation in vivo. (A) Immunoblot analysis of RNFT2, IL3Rα, and IL3Rβ from mice intratracheally treated with control or LPS, as indicated. Data and means ± SEM of 4 mice per group. (C) Cell concentration, (D) total protein analysis, (E-G) ELISA analysis of (E) IL-6 (F), TNF, (G) IL-1β from BAL fluid of mice intratracheally treated with Lenti-Empty or Lenti-RNFT2 and then treated with LPS and PBS or recombinant IL3, as indicated. Data and means ± SEM pooled of 3 mice per group are from 2 independent experiments. (H) Cell concentration, (I) total protein analysis, (J) ELISA analysis of IL-6 from BAL fluid of mice intratracheally treated with Lenti-control shRNA or Lenti-RNFT2 shRNA and then treated with LPS and PBS or recombinant IL3, as indicated. Data and means ± SEM pooled of 3 mice per group are from 2 independent experiments. (K-L) Histological analysis of lung samples from mice treated as indicated. Images are representative of all independent experiments. Scale bar, 100 μm. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, by Student’s t-test (B) or by one-way ANOVA with Tukey’s post hoc test (C-J).

Figure 6: The IL3Rα/RNFT2 signaling axis is relevant in human lung disease. (A) Immunoblot analysis of RNFT2, IL3Rα and tubulin from Human CF subjects lung explants with *Pseudomonas* colonization. Data and means ± SEM (n=12). (B) ELISA analysis of plasma IL3 in mechanically ventilated patients without lung injury (n=4) or At Risk for ARDS (n=16). Subject classification was blinded to the operator. Bars and error bars represent median and IQR, respectively. Data were analyzed by Mann Whitney U test. *P<0.05 by Mann-Whitney U Test. (C-D) Linear regression of plasma IL3 vs. plasma TNF (C) and IL1β (D) in ARFA subjects (n=16); *<P<0.05, ***P<0.001. (E-F) Linear regression of RNFT2 gene expression normalized to control patient vs. plasma TNF (E) and IL1β (F) in ARFA subjects (n=15).
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Abbreviations: SD: standard deviation; IQR: interquartile range; SOFA: sequential organ failure assessment; PaO2: partial pressure of arterial oxygen; FiO2: Fractional inhaled concentration of oxygen; WBC: white blood cell count; ICU LOS: intensive care unit length of stay;

**Table 1: Baseline characteristics of patients in the Acute Lung Injury Biospecimen Repository analyzed for plasma IL3 Levels.** Data are presented as mean (standard deviation) for normally distributed continuous variables or median (interquartile range) for not normally distributed continuous variables, and N (%) for categorical variables.
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<th>Variable</th>
<th>No Lung Injury</th>
<th>At Risk for ARDS (ARFA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>4</td>
</tr>
<tr>
<td>Age, mean (SD), yrs</td>
<td>64.9 (12.8)</td>
<td>51.8 (14.8)</td>
</tr>
<tr>
<td>Males, N (%)</td>
<td>4 (100)</td>
<td>10 (62.5)</td>
</tr>
<tr>
<td>SOFA score, median (IQR)*</td>
<td>5.0 (4.5-5.5)</td>
<td>7.0 (5.75-9.25)</td>
</tr>
<tr>
<td>PaO2:FIO2 ratio, mean (SD), mmHg</td>
<td>281.5 (187.7)</td>
<td>217.1 (101.5)</td>
</tr>
<tr>
<td>WBC, mean (SD), x 10^9 per liter</td>
<td>14.5 (10.8)</td>
<td>13.6 (8.7)</td>
</tr>
<tr>
<td>ICU LOS, median (IQR), days</td>
<td>4.5 (3.75-7.25)</td>
<td>6.5 (4.75-9.75)</td>
</tr>
<tr>
<td>30 Day mortality, N (%)</td>
<td>1 (25)</td>
<td>2 (12)</td>
</tr>
</tbody>
</table>

Abbreviations: SD: standard deviation; IQR: interquartile range; SOFA: sequential organ failure assessment; PaO2: partial pressure of arterial oxygen; FiO2: Fractional inhaled concentration of oxygen; WBC: white blood cell count; ICU LOS: intensive care unit length of stay;

**Table 1: Baseline characteristics of patients in the Acute Lung Injury Biospecimen Repository analyzed for plasma IL3 Levels.** Data are presented as mean (standard deviation) for normally distributed continuous variables or median (interquartile range) for not normally distributed continuous variables, and N (%) for categorical variables.