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High Levels of Triggering Receptor Expressed in Myeloid Cells-Like Transcript-1 Positive, but Not Glycoprotein 1b$^+$, Microparticles Are Associated With Poor Outcomes in Acute Respiratory Distress Syndrome

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OBJECTIVES: To identify triggering receptor expressed in myeloid cells-like transcript-1 positive (TLT-1$^+$) microparticles (MPs) and evaluate if their presence is associated with clinical outcomes and/or disease severity in acute respiratory distress syndrome (ARDS).

DESIGN: Retrospective cohort study.

SETTING: ARDS Network clinical trials.

PATIENTS: A total of 564 patients were diagnosed with ARDS.

INTERVENTIONS: None.

MEASUREMENTS AND MAIN RESULTS: Using flow cytometry, we demonstrated the presence of TLT-1$^+$ platelet-derived microparticles (PMP) that bind fibrinogen in plasma samples from fresh donors. We retrospectively quantified TLT-1, glycoprotein (Gp) 1b, or $\alpha_{IIb}\beta_{IIIa}$ immunopositive microparticles in plasma samples from patients with ARDS enrolled in the ARMA, KARMA, and LARMA (Studies 01 and 03 lower versus higher tidal volume, ketoconazole treatment, and lisofylline treatment Clincial Trials) ARDS Network clinical trials and evaluated the relationship between these measures and clinical outcomes. No associations were found between Gp1b$^+$ MPs and clinical outcomes for any of the cohorts. When stratified by quartile, associations were found for survival, ventilation-free breathing, and thrombocytopenia with $\alpha_{IIb}\beta_{IIIa}{}^+$ and TLT-1$^+$ MPs (χ^2 $p < 0.001$). Notably, 63 of 64 patients in this study who failed to achieve unassisted breathing had TLT$^+$ PMP in the 75th percentile. In all three cohorts, patients whose TLT$^+$ MP counts were higher than the median had higher Acute Physiology and Chronic Health Evaluation III scores, were more likely to present with thrombocytopenia and were 3.7 times ($p < 0.001$) more likely to die than patients with lower TLT$^+$ PMP after adjusting for other risk factors.

CONCLUSIONS: Although both $\alpha_{IIb}\beta_{IIIa}{}^+$ and TLT$^+$ microparticles ($\alpha_{IIb}\beta_{IIIa}$, TLT-1) were associated with mortality, TLT-1$^+$ MPs demonstrated stronger correlations with Acute Physiology and Chronic Health Evaluation III scores, unassisted breathing, and multiple system organ failure. These findings warrant further exploration of the mechanistic role of TLT-1$^+$ PMP in ARDS or acute lung injury progression.

KEYWORDS: acute respiratory distress syndrome, fibrinogen, microparticles, platelets, triggering receptor expressed in myeloid cells-like transcript-1

Acute respiratory distress syndrome (ARDS) is a life-threatening inflammatory condition that presents as acute-onset hypoxemia with noncardiogenic pulmonary edema, and bilateral opacities on lung radiograph. Although the definition of ARDS has changed throughout the years, the Berlin

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KEY POINTS

Question: Are triggering receptor expressed in myeloid cells-like transcript-1 positive (TLT-1$^+$) platelet-derived microparticles (PMP) prominent in acute respiratory distress syndrome (ARDS) and do they correlate with clinical outcomes and/or disease severity?

Findings: We retrospectively evaluated plasma from 564 patients diagnosed with ARDS and found that high levels of TLT-1$^+$ PMP had significantly higher correlations with mortality, multiple organ failure, and the inability to achieve unassisted breathing than glycoprotein 1b$^+$ and $\alpha_{IIb}\beta_{IIIa}^+$ PMP.

Meanings: The high correlation of TLT-1$^+$ PMP compared with other PMP subpopulations to mortality and other negative clinical outcomes suggests a role for TLT-1$^+$ PMP in ARDS.

Criteria specifies an arterial to inspired oxygen ratio (Pao_2/Fio_2) of less than 300 (1). ARDS is often complicated by thrombocytopenia and dysregulated hemostasis. The incidence of ARDS varies between countries from 10 to 82 per 10,000 and is likely underestimated in most countries (2). However, with an estimated mortality of up to 40% and a very high cost for its management, the global burden of ARDS is profound. ARDS arises most commonly as a complication of aspiration of gastric fluids, transfusion, trauma, sepsis, pneumonia, and more recently COVID-19 infection. ARDS has a complex inflammatory cause that eludes simple pharmacologic and prognostic paradigms. Randomized multicenter clinical trials, including the ARMA, KARMA, and LARMA (Studies 01 and 03 Lower versus higher tidal volume, ketoconazole treatment and lisofylline treatment Clincial Trials) conducted by the U.S. ARDS Network (3–5), have established standards of care for ARDS, which focus on supportive care for symptoms and ventilation using low tidal volumes to restore oxygenation and mitigate ventilator-induced damage to the alveolar epithelium. The ARDS Network enrolled patients with acute lung injury (ALI) or ARDS (according to the American European Consensus Conference on ARDS) in the ARMA trial from 1996 to 1999 (6). A total of 861 patients were enrolled in the ARMA trial which compared initial ventilation using 12 mL/kg ideal body weight (IBW) tidal volume (and plateau pressure goal of ≤ 50 cm H_2O) to a lower initial tidal volume (6 mL/kg IBW, with a plateau pressure goal of ≤ 30 cm H_2O). Some patients in the ARMA trial were concurrently enrolled in the KARMA trial (n = 234) which, using a 2 × 2 factorial design, randomized patients to receive ketoconazole (400 mg/d for 21 d) versus placebo. Ketoconazole ultimately did not improve survival, and enrollment continued in the ARMA tidal volume trial without a study drug through 1999. In 1998, the ARDS Network launched the LARMA trial, in which patients were randomized using a 2 × 2 factorial design to, lisofylline (3 mg/kg IV with a maximum dose of 300 mg every 6 hr for up to 20 d) versus placebo after co-enrollment and randomization in the ARMA tidal volume study. As with ketoconazole, there were no differences in survival according to whether patients received lisofylline or placebo; however, survival was higher among patients receiving lower tidal volumes in all three cohorts.

Platelets mediate seemingly paradoxical functions that are central to the cause of ARDS, yet essential for the resolution of ARDS pathologies (7). Although platelets are best characterized for their central roles in thrombosis and hemostasis, upon activation and degranulation, platelets release an arsenal of compounds that inter-regulate the pathways of thrombosis and hemostasis with those of inflammation and that modulate the integrity of the vascular endothelium (8). One important mediator of platelet activity is triggering receptor expressed in myeloid cells-like transcript-1 (TLT-1), a 32-kDa receptor that is expressed primarily in platelets and megakaryocytes. TLT-1 is stored in platelet α-granules and brought to the surface of activated platelets (9, 10). A soluble form of TLT-1 (sTLT-1) can be detected in plasma following platelet activation and is commonly detected in the plasma of patients with inflammatory conditions (11–13). The extracellular domain of TLT-1 binds to fibrinogen and the intracellular immune-tyrosine inhibitory motif-containing domain has been found to interact with g-protein important in intracellular traffiicking proteins, receptor for activated C kinase 1, growth factor receptor-bound protein 2, and SRC homology phosphatase-2 (12, 14). TLT-1 induces platelet aggregation, modulates platelet interactions with neutrophils and endothelial cells, and enhances fibrinogen deposition in lung injury models (9, 12, 15).

Our group has shown that high plasma concentrations of sTLT-1 are associated with poor prognosis and hypercoagulable state in sepsis (9, 10) and with increased mortality and decreased ability to achieve unassisted breathing in ARDS. In the ARDS Network ARMA/KARMA/LARMA clinical trial cohorts, patients with sTLT-1 concentrations greater than 1200 pg/mL were nearly three times more likely to die than patients with lower sTLT-1 concentrations, regardless of ventilation tidal volume (16).

In addition to the changes in morphology and platelet molecular signatures that accompany platelet activation, platelet-derived microparticles (PMP)—phosphatidylserine-exposed extracellular vesicles ranging in size between 100 and 1000 nm—are released in conjunction with thrombocyte activation (17–20). PMP is also released from megakaryocytes (21). In the vasculature, PMP are the most abundant, constituting 70–90% of MP, with others deriving from granulocytes, endothelial cells, monocytes, and RBCs (18, 22, 23). Like their platelet parents, PMP have both stimulatory and inhibitory effects on the pathways of thrombosis, hemostasis, and inflammation. In preclinical models of tissue damage, PMP accelerated ARDS-like sequelae and pulmonary complications and had prothrombotic activities (23–26). PMP, have been correlated with increased incidence of disease and poor clinical outcomes in COVID-19 (27), cardiovascular diseases (28, 29), preeclampsia (30), and autoimmune conditions (31), but have been associated with positive or protective roles in studies of other inflammatory conditions (32, 33)and reviewed in (34). However, no prior studies have evaluated TLT-1$^+$ PMP or their clinical correlates in disease cohorts.

In this study, we use flow cytometry to determine if TLT-1$^+$ PMP can be detected in plasma harvested from freshly activated platelets from healthy donors and in stored plasma samples from patients with ARDS/ALI who were enrolled in the ARMA trial. Further, we investigated the relationship between TLT-1$^+$ PMP concentrations and clinical outcomes in the ARDS Network ARMA-only cohort and then validated our findings in the KARMA and LARMA patient cohorts.

MATERIALS AND METHODS

Human Subjects

All studies using human subjects were approved by the University of Puerto Rico (UPR) and Maryville College (MC) Institutional Review Board (IRB) and all procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation with the Helsinki Declaration of 1975. UPR IRB Protocol 1617-013 and MC IRB Protocol no. 170920.01- TLT-1 levels in ARDS patients were originally approved September 20, 2016, and UPR Protocol 1213-126 was approved July 19, 2020, and Translational studies on TLT-1 were originally approved February 22, 2014.

Preparation of Platelet-Derived Microparticles From Fresh Plasma Samples From Healthy Donors

Blood was taken from five healthy volunteers after informed consent and processed as published previously (12). To obtain PMP in platelet-rich plasma or washed platelets, platelets were activated for 10 minutes with thrombin according to published protocol (12). Samples were centrifuged at 4°C and 5000 rcf for 15 minutes to pellet platelets. Supernatants were again centrifuged for 30 minutes at 4°C and 16,000 rcf, and the PMP-containing pellet was resuspended in flow activated cell sorting (FACS) buffer (PBS 0.1% BSA, 0.1% sodium azide) (**Supplemental Fig. 1*A* [top]**, http://links.lww.com/CCX/B360).

Preparation of Platelet-Derived Microparticles From Stored Patient Samples

Deidentified samples from patients for this study were originally obtained during the ARDS Network Clinical Trials (ARMA, KARMA, or LARMA). The eligibility criteria for these trials were acute hypoxemia (measured by a decrease in Pao_2/Fio_2 to ≤ 300), the requirement for positive pressure ventilation, bilateral pulmonary infiltrates on front chest radiograph with edema, and pulmonary-capillary occlusion pressure less than or equal to 18 mm Hg or absence of left atrial hypertension. Exclusion criteria were: greater than 36 hours since onset (meeting inclusion criteria); age younger than 18 years; morbid obesity (1 kg body mass/cm); burns covering greater than 30% of body surface area; prior bone marrow or lung transplantation; prior participation in other trials for ARDs, ALI, or sepsis; pregnancy; comorbidities such as increased intracranial pressure, neuromuscular disease, sickle cell disease, malignancy, terminal medical condition (estimated 6-mo mortality ≥ 50%); and chronic respiratory or liver disease. Patients

were excluded from the trial if their physician refused or was unwilling to use full life support.

According to the trial protocols, samples of blood were obtained from patients on day 1 and day 3 of the respective trials. Plasma from those samples was frozen and shipped to the National Institutes of Health Biorepository, where samples were stored at –80°C. After Institutional Review Board approval (UPR IRB protocol 1617-013 and MC IRB Protocol no. 170920.01, Exemption 4), frozen plasma samples from 799 patients were sent to us and stored at –80°C until our analysis of sTLT-1 concentrations. There was sufficient volume to quantify microparticles in 564 of those patient samples. Platelet-derived microparticles were separated from thawed day 1 plasma samples by centrifugation at 4°C and 5000 rcf for 15 minutes to pellet any cellular debris. Supernatants were again centrifuged for 30 minutes at 4°C and 16,000 rcf, and the PMP-containing pellet was resuspended in FACS buffer (Supplemental Fig. 1*A* [bottom], http://links.lww.com/CCX/B360).

Staining of Platelet-Derived Microparticles

In preparation for flow cytometric analysis, PMP derived from activated platelets or from frozen patient samples were stained with conjugated monoclonal antibodies for glycoprotein (GP) 1b, CD41, and TLT-1 using CD62-FITC- (clone AK-4, cat no. 5555523; BD Sciences, Franklin Lakes, NJ), CD42b PE-Cy5 (Clone HIP1, cat no. 551141; BD Sciences), CD41-PE (clone HIP8, cat no.555467; BD Sciences), CD31-FITC (platelet and endothelial cell adhesion molecule 1 [PECAM-1], clone 390, cat no.14-0311-82; Thermo Scientific, Waltham, MA) and TLT-1-Alexa 647 labeled in the house (AB69) (16) with isotype controls (cat no. 555750PE-Cy5 mouse IgG1 κ isotype control, cat no. 555748-FITC mouse IgG1, κ isotype control cat 556650 PE mouse IgG1, κ isotype control, mouse IgG2b Alexa 647 labeled in house, or FITC rat IgG2a κ isotype control [eBR2a], cat no. 11-4321-80; Thermo Scientific). Upon staining, PMP were analyzed by flow cytometry (Supplemental Fig. 1*A*–***C***, http://links.lww.com/CCX/B360).

Flow Cytometry

Flow cytometry was conducted on an Acuri C6 (BD Sciences). We used nano fluorescent beads 0.75–0.99 μm particle beads (cat no. NFPPS-0852-5; Spherotech, Lake Forest, IL) for calibration. The particle size gate was determined based on forward and side scatter of beads that were 0.75–0.99 μm, (upper limit of events measured). For each marker, we counted events within the gating area with fluorescence that exceeded that isotype control (**Supplemental Fig. 1*B***, http://links.lww.com/CCX/B360).

Fibrinogen Binding

PMP derived from thrombin-activated fresh plasma were incubated with 80 μg FITC-fibrinogen (Innovative Research, Novi, MI). Flow cytometry assays were performed on a BD FACSAriaTM III flow cytometer (BD Biosciences, San Jose, CA), with 50,000 positive events collected per sample, within 1 hours. Data were analyzed using FlowJo software, v10.6.1 (BD Biosciences), and changes in platelet population were evaluated using forward scatter versus Side Scatter parameters for all the conditions examined (Supplemental Fig. 1*B*, http://links.lww.com/CCX/B360).

Statistical Analysis

Diagnostic for normality criteria was performed using the Shapiro-Francia estimator. The presence of outliers was verified via Grubbs test. Raw data distribution were assessed using central tendency and dispersion measures. Bivariate schemata were evaluated with the Kruskal-Wallis test with post hoc assessment. Zero-point correlations between continuous variables were evaluated using the Spearman test. Proportion comparisons were evaluated using the chi-square test of independence. Binary logistic regression was used to determine the likelihood of an event after ruling out multicollinearity phenomena (variance inflation factor < 1.5 for all independent variables). Receiver operator characteristics (ROCs) and Kaplan-Meier log-rank analysis were used to determine the predictive value of indicators and survival, respectively. IBM-SPSS (Armonk, NY) 27 was used in all computations. The significance level (α) was set to less than or equal to 0.05, except for the normality diagnostic test ($p > 0.05$).

RESULTS

Identification of TLT-1$^+$ Microparticles in Plasma Samples From Healthy Donors

Platelet microparticles are the most commonly found in the blood (18, 22, 23), and we hypothesized that activated platelets release TLT-1$^+$ PMP. Using the strategy

outlined in Supplemental Figure 1*A* (top) (http://links.lww.com/CCX/B360), we identified PMP from resting and activated platelets. Freshly collected plasma samples provided from five healthy volunteers were stained for TLT-1. Staining for TLT-1 is markedly enhanced in PMP derived from activated platelets. We subjected MP derived from either resting or activated platelets to fluorescently labeled fibrinogen to measure bound fibrinogen on TLT-1$^+$ PMP using flow cytometry. We demonstrate the existence of platelet-derived TLT-1 immunopositive microparticles. Fibrinogen staining is increased on TLT-1$^+$ MP isolated from activated platelets compared with resting platelets or controls (Supplemental Fig. 1*B*, http://links.lww.com/CCX/B360).

Evaluation of TLT-1$^+$ Microparticles in ARDS Patients

PMP were harvested from stored plasma samples from ARDS patients and quantified by flow cytometry following the strategy outlined in Supplemental Figure 1*A* (bottom) (http://links.lww.com/CCX/B360). **Table 1** shows the characteristics of the 564 patients from whom PMP were quantified in this study, according to the trials in which they were enrolled, as well as the healthy volunteers. The median age of all the ARDS/ALI patients in the samples analyzed for this study was 48 years (range, 18–87), and 41% were female. The Average Acute Physiology and Chronic Health Evaluation III score was 84 (range, 47–182). Just over

TABLE 1.
Volunteer and Patient Characteristics (Descriptive Schema)

Characteristic	Volunteers, *n* = 5 (%)	All Patients, *n* = 564 (%)	ARMA Cohort, *n* = 258 (%)	KARMA Cohort, *n* = 151 (%)	LARMA Cohort, *n* = 155 (%)
Female (%)	2 (40%)	232 (41%)	120 (47%)	60 (40%)	52 (34%)
Average age (range) (yr)	36.4 (27–55)	48.1 (18–87)	47 (18-84)	51 (19-87)	47 (18-86)
Average Acute Physiology and Chronic Health Evaluation III Score	NA	84 (47–182)	82 (30–172)	83 (39–147)	90 (27–182)
Race					
White	1 (20%)	423 (75%)	197 (76%)	106 (70%)	120 (78%)
African American	1 (20%)	94 (17%)	37 (14%)	34 (23%)	23 (15%)
American Indian/Alaska Native		45 (8%)	24 (9%)	11 (7%)	10 (7%)
Hispanic	3 (60%)				
Cause					
Sepsis	NA	212 (38%)	84 (33%)	70 (47%)	58 (38%)
Trauma	NA	71 (13%)	37 (14%)	19 (13%)	15 (10%)
Aspiration	NA	114 (20%)	51 (20%)	31 (21%)	32 (21%)
Pneumonia	NA	252 (45%)	130 (51%)	45 (48%)	77 (51%)
Tidal volume (mL/kg)					
6	NA	313 (56%)	128 (50%)	93 (62%)	92 (60%)
12	NA	251 (45%)	130 (50%)	58 (38%)	63 (41%)
Study drug					
No drug	NA	258 (46%)	258 (100%)	–	–
Ketoconazole	NA	73 (13%)	–	73 (48%)	–
Lysofilline	NA	76 (14%)	–	–	76 (49%)
Placebo	NA	157 (28%)	–	78 (52%)	79 (51%)

ARMA = Lower Tidal Volume Trial, KARMA = ketoconazole for [Acute Lung Injury/Acute Respiratory Distress Syndrome], LARMA = lisofylline for [Acute Lung Injury/Acute Respiratory Distress Syndrome], NA = not applicable.
Dashes indicate no data.

half received the 6-mL/kg IBW tidal volume, and 46% had been enrolled in the ARMA trial only comparing tidal volumes. Among the patients in the KARMA and LARMA cohorts, half received the study drug (ketoconazole or lysophiline, respectively), and half placebo. Although the mean platelet count at baseline was within the normal range, 57% of the patients in the trial were thrombocytopenic at baseline, and 26% presented with platelet counts less than 80,000 platelets/μL. Neither platelet counts nor plasma sTLT-1 concentrations (mean [range], 1761 [0–21,864] pg/mL) varied significantly with age, gender, ventilation volume, drug type, or baseline Pao_2/Fio_2. Pneumonia was the most common cause of ALI/ARDs among the patients in the cohort.

Platelet surface markers were measured on the PMP from day 1 plasma samples by flow cytometry. A representative patient sample is depicted in **Supplemental Figure 1*C*** (http://links.lww.com/CCX/B360). We considered several choices of platelet receptors including PECAM and P-selectin. We chose not to measure PECAM because it is also expressed in some plasma cells, monocytes, immature dendritic cells, neutrophils, natural killer cells, and lymphocytes. Although we did measure P-selectin, it is not reported here because P-selectin is also found on endothelial cells and there was no significant association with its expression and mortality, days of hospitalization, or time to unassisted breathing.

Using 258 patient samples from patients enrolled in the ARMA trial only (no study drug), we investigated associations between the PMP biomarkers TLT-1, GP1b, and $\alpha_{IIb}\beta_{IIIa}$, and between PMP counts and platelet counts, and plasma concentrations of sTLT-1. Average total TLT-1$^+$, GP1b$^+$, and $\alpha_{IIb}\beta_{IIIa}{}^+$ event counts (± SD) were 168 ± 181, 64 ± 92, and 35 ± 250 events/μL respectively. The average plasma sTLT-1 concentration was 1627 ± 1687 pg/mL and platelet counts were 149,000 ± 115,000/μL in the patient samples. Total TLT-1$^+$ PMP counts correlated with total Gp1b counts and $\alpha_{IIb}\beta_{IIIa}$ counts, (ρ = 0.577 and 0.610, respectively, $p < 0.001$) (**Supplemental Fig. 2*A***, http://links.lww.com/CCX/B360). Correlations were weak between total TLT-1$^+$ PMP and sTLT-1 (ρ = 0.114, $p < 0.068$) (Supplemental Fig. 2*A*, http://links.lww.com/CCX/B360) but were stronger between TLT-1$^+$ PMP and platelets (ρ = –0.316, $p < 0.001$). The same trends were seen in samples from the KARMA and LARMA cohorts (data not shown).

In the ARMA cohort, total TLT-1$^+$, but not GP1b$^+$ or $\alpha_{IIb}\beta_{IIIa}{}^+$, PMP counts were higher for patients who succumbed to ARDS than for survivors ($p < 0.001$) (**Fig. 1*A*** and **Table 2**). Likewise, total TLT-1$^+$ PMP counts were higher among patients who failed to achieve independent breathing and those who developed multisystem organ failure (Table 2). When we analyzed the study endpoints according to quartiles of PMP count, ARMA patients with the highest TLT-1$^+$ PMP had the worst clinical outcomes with 65% of patients who died and all but 1 of those who failed to achieve ventilation-free breathing being in the fourth quartile for TLT-1$^+$ PMP (Fig. 1*B*), (χ^2 $p < 0.01$). We repeated these analyses using the KARMA and LARMA cohorts and saw the same magnitudes and levels of significance in both cohorts (not shown).

We tested whether median PMP counts could serve as a useful binary cutoff for predicting survival among patients in the ARMA cohort using the ROC (**Supplemental Fig. 2*B***, http://links.lww.com/CCX/B360). ROC showed that TLT-1$^+$ PMP was a much stronger predictor of survival than the other PMP biomarkers evaluated here, with an AUC of 0.749 ($p < 0.00001$) for TLT-1$^+$ PMP. With a cutoff of PMP $\times 10^3$/μL (median), the sensitivity was 81% and specificity was 57% in the ARMA cohort; the same trends were observed in ARMA and KARMA cohorts using median biomarker-positive PMP count as a cutoff, with TLT-1$^+$ PMP having highest sensitivity and specificity in each cohort (not shown). In each cohort TLT-1$^+$ PMP were associated with higher AUC in ROC than plasma TLT-1 (not shown).

Correlation of Microparticles to Clinical Outcomes of Patients With ARDS

We then evaluated differences in hematologic, clinical, and therapeutic parameters that are associated with ARDS prognosis for patients in the ARMA cohort using median TLT-1$^+$ PMP counts as a bivariate cutoff (**Table 3**). Although there was no difference in the proportion of patients receiving higher tidal volume, between the two groups, patients with TLT-1$^+$ PMP higher than the median had higher plasma sTLT-1 concentration, lower platelet counts, and were more likely to present with thrombocytopenia and had higher Acute Physiology and Chronic Health Evaluation (APACHE) III scores ($p < 0.001$) in all three trial cohorts. Finally, using the median TLT-1$^+$ PMP as a cutoff, found that patients

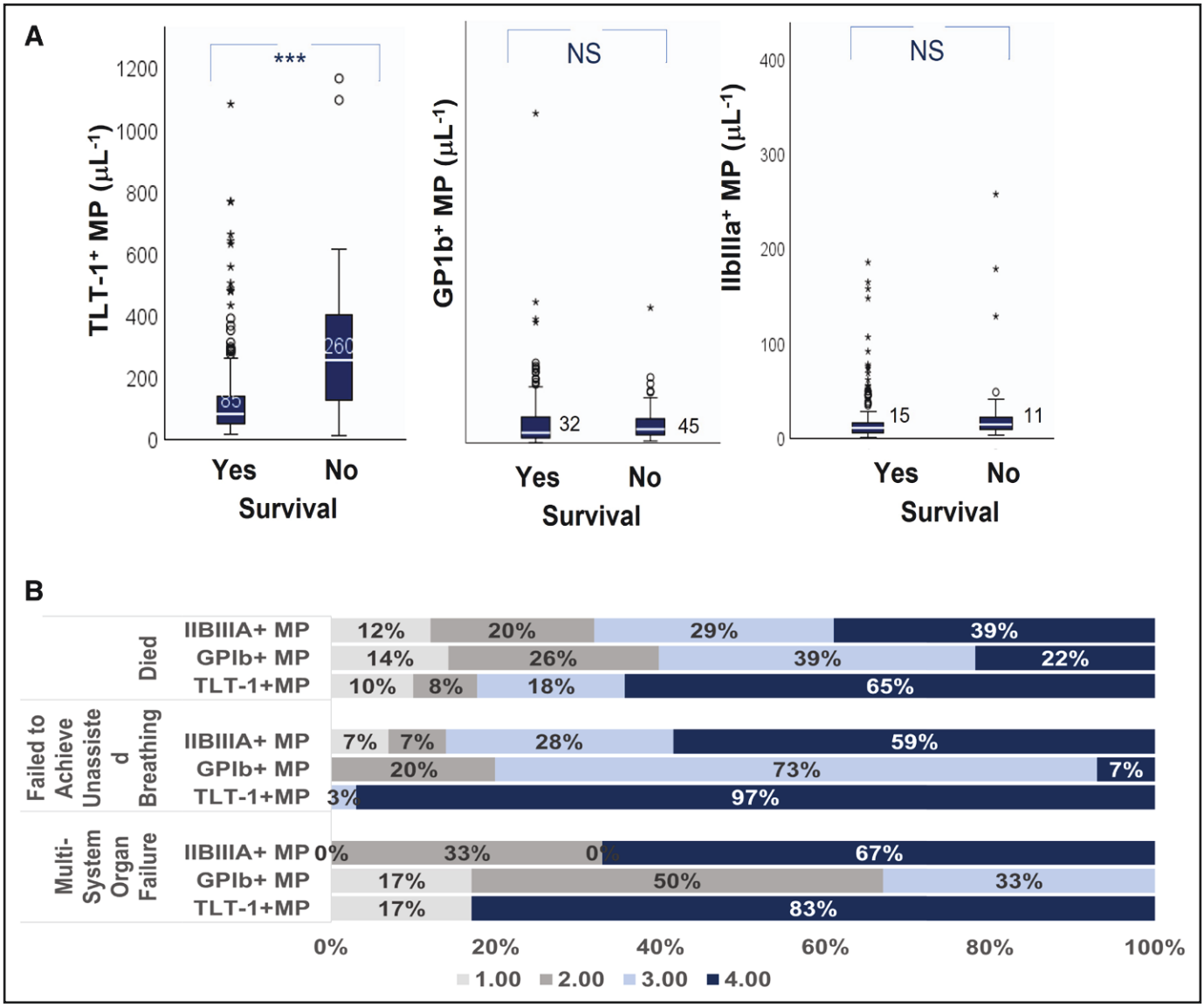

Figure 1. High TREM-like transcript-1 positive (TLT-1+) platelet microparticle (MP) counts correlate with poor clinical outcomes. **A**, Compares platelet-derived microparticles (PMP) expression of TLT-1, GP1b, and/or $\alpha_{IIb}\beta_{IIIa}$ for survivors and nonsurvivors. Totals were compared by analysis of variance. ***$p < 0.01$. **B**, Proportions of patients experiencing poor clinical outcomes according to PMP counts (by quartile). Chi-square $p < 0.001$ for TLT-1+ PMP for all outcomes. $n = 258$ for both graphs. GP1b = glycoprotein 1b, NS = not significant, TREM = triggering receptor expressed in myeloid cells.

with higher TLT-1+ PMP had shorter survival time ($p < 0.001$) (**Supplemental Fig. 2*C***, http://links.lww.com/CCX/B360), were more likely to die, and were less likely to achieve independent breathing in the ARMA cohort (**Table 4**). These trends were maintained in the two other trial cohorts. After adjusting for other significant predictors of survival (plasma sTLT-1+ concentration, thrombocytopenia, ventilation volume, APACHE III score) the odds ratio (OR) for mortality was 4.3 (95% CI, 2–6.9; $p < 0.001$) for the ARMA patients whose TLT-1+ PMP exceeded $\times 10^3/\mu L$ (**Table 5**). Notably, TLT-1+ PMP was associated with higher OR and stronger correlation than plasma sTLT-1 concentration. Using the same adjustment strategy, and a binary cutoff at the median for each of the other two trial cohorts, the OR for mortality for TLT-1+ PMP was of similar magnitude and significance in the KARMA cohort but was lower and fell just below the criteria for significance for the LARMA cohort.

DISCUSSION

We demonstrate, using flow cytometry, the existence of TLT-1+ platelet microparticles in fresh plasma samples after platelet activation, and in frozen plasma samples

TABLE 2.

Comparison of Plasma Soluble Triggering Receptor Expressed in Myeloid Cells-Like Transcript-1 Concentrations, Platelet Counts, and Platelet-Derived Microparticles Counts (Mean ± SD) for Patients in the Lower Tidal Volume Trial (No Study Drug) According to Study Endpoints

	Survived		Achieved 48-hr Unassisted Breathing		Multisystem Organ Failure	
	Yes (207)	No (51)	Yes (229)	No (29)	Yes (6)	No (252)
Plasma (soluble TLT-1), pg/mL	1527 ± 1694[a]	2031 ± 1609[a]	1591 ± 1725[a]	1913 ± 1335[a]	2408 ± 2125	1608 ± 1676
Platelets (× 10^3), μL^{-1}	168 ± 116[a]	73 ± 70[a]	164 ± 114[a]	35 ± 14[a]	42 ± 6[a]	152 ± 115[a]
TLT-1 positive PMP, μL^{-1}	111 ± 116[a]	274 ± 227[a]	113 ± 115[a]	384 ± 231[a]	296 ± 198[a]	140 ± 156[a]
GP1b$^+$ PMP, μL^{-1}	40 ± 48	41 ± 49	42 ± 51	28 ± 11	18 ± 9	41 ± 49
$\alpha_{IIb}\beta_{IIIa}{}^+$ PMP, μL^{-1}	31 ± 271[a]	19 ± 35[a]	29 ± 258[a]	27 ± 45[a]	13 ± 7	29 ± 246

GP1b = glycoprotein 1b, PMP = Platelet-Derived Microparticles, TLT-1 = triggering receptor expressed in myeloid cells-like transcript-1.
[a]Significant for survival on Kruskal-Wallis.

TABLE 3.

Comparison of Clinical Characteristics of Acute Respiratory Distress Syndrome Patients Stratified by Triggering Receptor Expressed in Myeloid Cells-Like Transcript-1 Positive Platelet-Derived Microparticles (Cutoff at Median, ×10^3/μL) Among Patients in the Lower Tidal Volume Trial Only Cohort (No Study Drug)

	TLT-1$^+$ PMP ≤ ×10^3/μL, *n* = 129	TLT-1$^+$ PMP > ×10^3/μL, *n* = 129	*p*
Plasma (soluble TLT-1), pg/mL	1542 ± 1644	1712 ± 1730	0.17
Platelets, ×10^3/μL	176 ± 110	122 ± 114	< 0.001
Age, yr	46 ± 15	51 ± 16	0.014
Acute Physiology and Chronic Health Evaluation III Score	71 ± 25	92 ± 27	< 0.001
Pao_2/Fio_2	137 ± 59	135 ± 63	NS
12-mL/kg ideal body weight tidal volume	64 (50%)	64 (50%)	NS

NS = not significant, PMP = Platelet-Derived Microparticles, TLT-1$^+$ = triggering receptor expressed in myeloid cells-like transcript-1 positive.
p values are based on Kruskal-Wallis analysis for continuous variables or χ^2 analysis for proportions.

TABLE 4.

Clinical Outcomes Stratified by Triggering Receptor Expressed in Myeloid Cells-Like Transcript-1 Positive Platelet-Derived Microparticles Counts (Cutoff at the Median, ×10^3/μL) Among Patients in the Lower Tidal Volume Trial (No Study Drug)

	TLT-1$^+$ PMP ≤ ×10^3/μL, *n* =129	TLT-1$^+$ PMP > ×10^3/μL, *n* = 129	*p*
Failed to achieve unassisted breathing	0	29 (23%)	< 0.001
Developed multisystem organ failure	1 (0.8%)	5 (4%)	NS
Died	9 (7%)	42 (33%)	< 0.001
Mean survival days[a] (95% CI)	174(168–179)	134 (121–147)	< 0.001

TLT-1+ = triggering receptor expressed in myeloid cells-like transcript-1 positive.
[a]*p* values are based on Kruskal-Wallis analysis for continuous variables or χ^2 analysis for proportions.

TABLE 5.

Odds Ratios for Survival Stratified by Triggering Receptor Expressed in Myeloid Cells-Like Transcript-1 Positive Platelet-Derived Microparticles Stratification (Cutoff at the Median) in Acute Respiratory Distress Syndrome Patients Lower Tidal Volume Trial, Ketoconazole for [Acute Lung Injury (ALI)/Acute Respiratory Distress Syndrome (ARDS)], Lisofylline for ALI/ARDS Cohorts

	ARMA, ×103/μL Cutoff		KARMA, ×108/μL Cutoff		LARMA, ×98/μL Cutoff	
	OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*
Mortality						
Adjusted[a]	4.3 (1.8–10.3)	< 0.001	4.4 (1.7–11.8)	< 0.001	3.0 (1.0–9.2)	0.051
Unadjusted	6.5 (3.0–13.9)	< 0.001	4.9 (2–12.3)	< 0.001	3.8 (1.3–11.0)	0.068

ARMA = Lower Tidal Volume Trial, KARMA - ketoconazole for ALI/ARDS, LARMA - lisofylline for ALI/ARDS, OR = odds ratio.
[a]Binary logistic analysis controlled for soluble triggering receptor expressed in myeloid cells-like transcript-1 thrombocytopenia, Acute Physiology and Chronic Health Evaluation III score, and ventilation tidal volume.

collected from patients with ARDS. Further, we demonstrate increased fibrinogen binding for TLT-1$^+$ MP derived from activated vs. resting platelets from healthy volunteers. In proteomic analysis, TLT-1 peptides have been detected in microparticles derived from adenosine diphosphate-activated platelets from fresh donors (35, 36) and from plasma of patients with coronary artery disease (37, 38). Other proteomic studies did not identify TLT-1 peptides in extracellular vesicles from thrombin-activated platelets or platelets isolated from obese patients (39–41). This discordance could be attributed to differences inpatient population, sample collection and storage methods, or PMP enrichment strategies, all of which have been shown to affect the accuracy of PMP counts (20). These studies confirm the physical presence of TLT-1$^+$ PMP.

In our analysis of the plasma samples from an ARDS cohort, TLT-1$^+$ PMP were much more abundant than GP1b, $\alpha_{IIb}\beta_{IIIa,}$ or P-selectin. This is consistent with prior reports that TLT-1 is abundant on activated platelets (copy number ~55,000 on activated platelets) and with the observation that the mean sTLT-1 concentration in the set of samples quantified in this study was greater than 1200 pg/mL, indicative of platelet activation. The absence of strong correlations between PMP for the platelet biomarkers in this study is consistent with observations that PMP harvested from plasma are heterogenous, deriving from different activation pathways. However, the negative correlation between TLT$^+$ PMP and platelet counts is consistent with observations of negative correlations between platelet counts and soluble or PMP-associated markers of platelet activation (42, 43).

One obvious weakness of our study is the retrospective analysis using plasma samples that had been preserved for many years. Even though the samples were meticulously preserved by the NIH biorepository we were not responsible for collection or their initial storage. Given the known variability in PMP quantifications associated with different collection, storage, and detection methods, more studies will be needed and more robust selection and quantification criteria before TLT$^+$ PMP might be considered a reliable biomarker in ARDS. Nevertheless, all samples in this analysis were treated the same, so the differences we measured are indicative of differences that existed in the preserved samples.

High TLT-1$^+$, but not GP1b$^+$ or $\alpha_{IIb}\beta_{IIIa}{}^+$, PMP counts are associated with poor clinical outcomes in patients with ARDS/ALI. ROC analysis TLT-1$^+$ PMP counts to be a stronger predictor of survival than $\alpha_{IIb}\beta_{IIIa}{}^+$ or Gb1b$^+$ PMP counts and better than plasma sTLT-1 concentrations in these patient cohorts. In this analysis, patients whose plasma samples had TLT$^+$ PMP counts in the upper half of the distribution (and particularly in the 75th percentile) had higher APACHE scores, lower 180-day survival rates, and shorter survival times and were less likely to achieve ventilator-free breathing than patients with lower TLT-1$^+$ PMP counts. These correlations are stronger than soluble TLT-1 or platelet counts. TLT-1$^+$ PMP counts were the strongest predictor of survival, with an OR for survival of 4.3 (95% CI, 1.8–10.3; $p < 0.001$) versus 2.6 for

sTLT-1 (95% CI, 1.1–5.5; $p = 0.016$) after adjusting for age, APACHE III score, ventilation volume, creatinine, and thrombocytopenia. These consistent differences in clinical outcomes between patients whose plasma samples had elevated TLT-1 PMP and those with lower counts were both statistically significant and clinically meaningful and therefore warrant additional exploration of TLT-1$^+$ PMP as a biomarker for inflammatory conditions like ARDS and continued investigation into the role of TLT-1 in the cause of ARDS. Our studies demonstrating TLT-1$^+$ microparticles are supported by the recent work of Tyagi et al (44), who identified TLT-1$^+$ microparticles in the context of lung cancer and the suppression of CD8$^+$ T cells. Tyagi et al (44) demonstrated that interactions of sTLT-1 with CD3ε inhibit T cell function, it would be interesting to see if the (s)TLT-1 and or TLT-1$^+$ PMP in ARDS inhibit T cell function thereby increasing disease severity. As such, blocking TLT-1 may be an intervention for ARDS as well as certain types of cancer such as the lung.

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