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BRIEF REPORT

Evaluation of a new bead-based assay to measure levels of human tissue factor antigen in extracellular vesicles in plasma

Sierra J. Archibald BS[1] | Yohei Hisada PhD[1] | Victoria L. Bae-Jump MD, PhD[2,3] | Nigel Mackman PhD[1]

[1]Division of Hematology, UNC Blood Research Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

[2]Division of Gynecologic Oncology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

[3]Lineberger Comprehensive Cancer Center, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

Correspondence
Nigel Mackman, Division of Hematology, Department of Medicine, University of North Carolina at Chapel Hill, 116 Manning Drive, Mary Ellen Jones Building, Room 8004B, Campus Box 7035, Chapel Hill, NC, 27599.
Email: nigel_mackman@med.unc.edu

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Abstract

Background: Circulating tissue factor (TF)-expressing extracellular vesicles (EVs) are associated with thrombosis in several diseases, such as coronavirus disease 2019 (COVID-19). Activity assays have higher sensitivity and specificity compared to antigen assays for measuring TF+ EVs in plasma. The MACSPlex Exosome Kit is designed to detect 37 exosomal surface epitopes, including TF, on EVs in plasma using various fluorescently labeled beads. The different EV-bead complexes are detected by flow cytometry. A recent study used the MACSPlex Exosome Kit to measure levels of TF+ EVs in serum from patients with COVID-19.

Objectives: To evaluate the ability of the MACSPlex Exosome Kit to detect TF on EVs in plasma.

Methods: We measured levels of TF+ EVs isolated from plasma with or without TF detected using our in-house EVTF activity assay and the MACSPlex Exosome Kit.

Results: The MACSPlex Exosome Kit gave a very low TF antigen signal (TF bead signal) compared to platelet-derived CD41b+ EVs, which was used as a control. Lipopolysaccharide (LPS) increased levels of EVTF activity but not TF bead signal in four donors. Inhibition of TF reduced levels of EVTF activity but did not affect the TF bead signal in EVs isolated from plasma from LPS-treated blood. Finally, we found no correlation between levels of EVTF activity and TF bead signal in EVs isolated from plasma from ovarian cancer patients ($r = .16$, $P = .62$).

Conclusion: Our data suggest that the MACSPlex Exosome Kit gives a nonspecific signal for TF and does not have the sensitivity to detect TF+ EVs in plasma.

KEYWORDS
biomarker, COVID-19, extracellular vesicle, thrombosis, tissue factor

Essentials

- Tissue factor (TF) antigen-based assays have low sensitivity and specificity compared with activity-based assays.
- The MACSPlex system was developed to measure levels of 37 antigens, including TF, on exosomes.
- We evaluated the ability of the MACSPlex system to detect TF on extracellular vesicles (EVs) isolated from plasma.
- The MACSPlex system did not detect TF-positive EVs in plasma.

1 | INTRODUCTION

Tissue factor (TF) is expressed by various cell types and is required for hemostasis.[1] However, under pathological conditions, it is expressed by different cell types, including monocytes and cancer cells, and released into the circulation on extracellular vesicles (EVs).[1] Increased levels of TF+ EVs in the circulation are associated with activation of coagulation and thrombosis in different diseases, such as cancer, sepsis, and coronavirus disease 2019 (COVID-19).[2-7] Levels of TF+ EVs can be measured using either activity-based or antigen-based assays.[8] Activity-based assays are more sensitive than antigen-based assays.[9] In addition, antigen-based assays cannot distinguish between active versus encrypted TF. We and others have developed assays to measure EVTF activity of EVs isolated from plasma.[10-13] There is also a commercial assay called ZYMUPHEN MP-TF assay (Aniara, West Chester, OH, USA) that captures TF+ EVs using an anti-TF antibody and then measures their TF activity. We found that this commercial assay was less specific than our in-house assay due to the use of a high concentration of factor VIIa.[14] We have also captured TF+ EVs from plasma using an anti-TF antibody and detected them using a fluorescently labeled annexin V and laser scanning confocal microscopy.[15]

Miltenyi Biotec (Bergisch Gladbach, Germany)developed a MACSPlex Exosome Kit that contains 39 different antibodies, which includes two isotype controls, each bound to different fluorescently labeled beads.[16] One of the beads contains the anti-TF antibody called REA949, which is a clone of the monoclonal anti-human TF antibody HTF-1.[17] EVs are isolated from plasma samples and incubated with the beads to form EV-bead complexes. These complexes are then detected using three allophycocyanin (APC)-labeled exosome markers (CD9, CD63, and CD81) by flow cytometry. A recent study used the MACSPlex Exosome Kit to measure levels of TF+ EVs in serum from individuals who were positive and negative for severe acute respiratory syndrome coronavirus 2 and healthy controls.[18] It should be noted that the EVs were not isolated from the serum as recommended by Miltenyi Biotec. The study concluded that levels of TF+ EVs were increased in patients with COVID-19. We and others have reported increased levels of EVTF activity in patients with COVID-19 compared with controls and that increased levels are associated with increased thrombosis and decreased survival.[2-4,6]

In this study, we evaluated the ability of the MACSPlex Exosome Kit to capture TF+ EVs isolated from plasma prepared from lipopolysaccharide (LPS)-treated whole blood from healthy donors and patients with ovarian cancer.

2 | METHODS AND MATERIALS

2.1 | Preparation of plasma samples with or without TF

A protocol to collect blood from healthy volunteers who provided written consent was approved by the University of North Carolina at Chapel Hill Institutional Review Board (14-2108). Blood was collected into sodium citrate tubes as described.[5] Platelet-free plasma was prepared immediately from a portion of the blood as a TF-negative control by centrifuging whole blood without brake twice at 2500 *g* for 15 minutes at room temperature. Another portion of the blood from each donor was treated with LPS (10 μg/mL; Sigma-Aldrich, St. Louis, MO, USA; Cat. no. L2630) for 5 hours at 37°C with gentle shaking followed by plasma preparation to generate a TF-positive sample. Serum was prepared by treating plasma with calcium chloride (2 mg/mL) overnight and collecting the supernatant.

2.2 | Samples from patients with ovarian cancer

Blood samples were collected from patients with ovarian cancer who provided written consent using a protocol approved by the University of North Carolina at Chapel Hill Institutional Review Board (11-1201). Platelet-poor plasma was prepared by centrifuging blood without brake at 1500 *g* for 15 minutes at room temperature.

2.3 | EV TF activity assay

We measured levels of EVTF activity in plasma and serum samples using an in-house assay.[12]

2.4 | Measurement of TF antigen + EVs in plasma samples

We used the MACSPlex™ Exosome Kit (Miltenyi Biotec; Cat. no. 130-122-209) to measure levels of TF+ EVs in plasma samples as described by the manufacturer with some modifications. EVs in plasma were pelleted by centrifugation at 20,000 *g* for 15 minutes at room temperature. EVs were washed with N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffered saline (pH 7.4: 137 mmol/L NaCl, 5.38 mmol/L KCl, 5.55 mmol/L glucose, 10 mmol/L HEPES)

containing bovine serum albumin (0.1% w/v) and then repelleted using the same condition before being resuspended in phosphate-buffered saline. TF antigen+ EV-bead complexes and CD41b+ EV-bead complexes were detected using APC-labeled exosome markers (CD9, CD63, and CD81). Mean fluorescence intensity (MFI) was measured using a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec). We subtracted the average of the two controls (REA and mIgG1) from the bead intensity values for the TF (TF bead signal) and CD41b (CD41b bead signal) beads. We did not normalize to the average of the three exosome markers (CD9, CD63, and CD81). Samples were run in singlet or quadruplet. In one experiment we used an APC-labeled anti-TF antibody (REA949, Miltenyi Biotec, Cat. no. 130-115-685) to detect TF+ EVs complexed with beads.

2.5 | Statistical Analysis

The Shapiro-Wilk test was used for normality testing. Data are shown as mean + standard deviation for normally distributed data or median ± interquartile range for nonnormally distributed data. The correlation between TF bead signal and EVTF activity was tested using the Spearman's rank correlation coefficient. The statistical analyses were performed with Prism version 7.03 (GraphPad Software, La Jolla, CA, USA). Values of $P < .05$ were considered statistically significant.

3 | RESULTS AND DISCUSSION

The study by Balbi et al.[18] measured TF bead signal in discarded serum samples rather than plasma from patients with COVID-19 using the MACSPlex Exosome Kit. In addition, beads were added to serum rather than isolating the EVs and then incubating them with the beads as recommended by Miltenyi Biotec. We hypothesized that the number of TF+ EVs would be greatly reduced in serum compared with plasma because they would be incorporated into the clot. Therefore, we compared levels of EVTF activity in two paired plasma and serum samples from whole blood from healthy donors stimulated with LPS. As expected, levels of EVTF activity were decreased by 74% (0.59–0.14 pg/mL) and 89% (3.31–0.38 pg/mL) in serum samples compared to plasma samples. This suggests that most of the TF+ EVs in plasma are incorporated into the clot during the preparation of serum. Therefore, it is better to use plasma rather than serum for measurement of TF+ EVs.

We prepared paired TF-negative and TF-positive plasma controls from whole blood from four healthy donors. As expected from our previous studies,[5,11,14] EVTF activity in the four donors was detectable only in the TF-positive controls and varied between 0.9 and 2.6 pg/mL, with donor 4 having the highest level (Figure 1A). In contrast, very low TF bead signals were observed in the TF-positive controls from donors 2 through 4, and only donor 1 had a detectable bead signal in the LPS sample using MACSPlex Exosome Kit (Figure 1B). Notably, the TF bead signal did not correspond to the

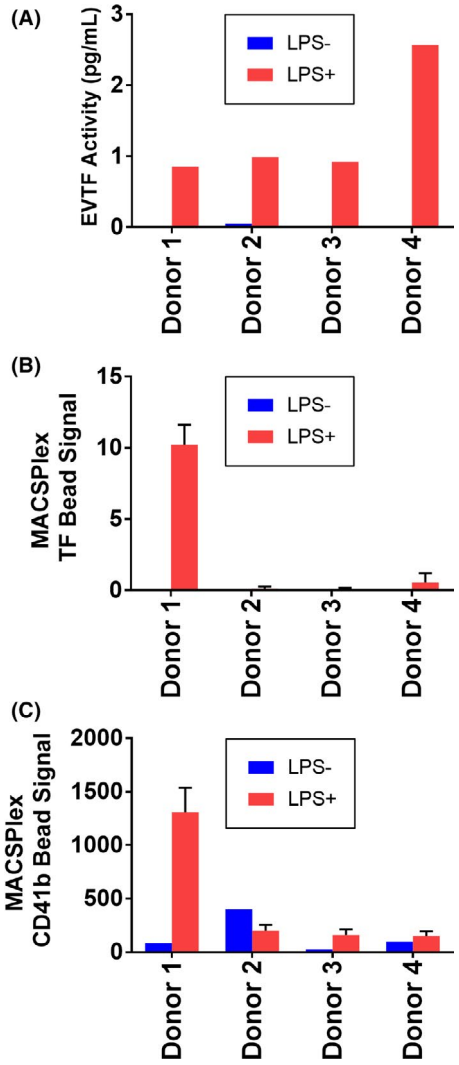

FIGURE 1 Comparison of levels of extracellular vesicle tissue factor (EVTF) activity and tissue factor bead signal. Extracellular vesicles (EVs) were isolated from plasma from whole blood from four healthy donors without (blue bars) or with (red bars) stimulation with lipopolysaccharide (LPS). (A) EVTF activity was measured using an in-house assay; (B) TF bead signal and (C) CD41b bead signal were measured using the MACSPlex Exosome Kit. EVTF activity was measured in singlet, TF antigen in negative LPS samples was measured in singlet, and TF antigen in positive LPS samples was measured in quadruplet. Mean + standard deviation is shown for the positive LPS data

EVTF activity. Next, we measured levels of CD41b+ EVs as a control for the MACSPlex Exosome Kit because platelet-derived EVs are abundant in plasma. A low signal was observed for CD41b in the negative control samples (Figure 1C). Three of the four donors had an increase in CD41b+ EVs in the LPS-treated samples (Figure 1C). Donor 1 had the highest TF bead signal and CD41b bead signal in the LPS-treated sample. Importantly, the CD41b bead signal was >100 times higher than the TF bead signal for donor 1. This suggests that the TF bead signal may be below the detection threshold of the assay. We also attempted to detect TF+ EV-bead complexes with an

APC-labeled anti-TF antibody REA949, but no signal was detected (data not shown).

An important way of demonstrating specificity of an assay is to show that blocking the target with an antibody reduces the signal. The MACSPlex Exosome Kit uses an anti-TF antibody called REA949, which is a clone of HTF-1 (Dr Stefan Wild, Miltenyi Biotec, Inc., personal communication). Therefore, we determined the effect of incubating the samples with HTF-1 (7.8 μg/mL for EVTF activity[12] and 15 μg/mL for TF bead signal[18]) before measuring levels of EVTF activity and TF bead signal. As expected, HTF-1 reduced factor Xa generation of the EVs from LPS-treated blood from all 4 donors by an average of 88% (Figure 2A). In contrast, HTF-1 did not affect the TF bead signal observed in donor 1 (Figure 2B). This suggests that the TF bead signal detected using the MACSPlex Exosome Kit is not specific for TF. In contrast to our results, Balbi et al.[18] found that pre-incubation of EVs in serum with either an anti-TF antibody or human recombinant factor VII reduced the TF bead signal.

Finally, we measured levels of EVTF activity and TF bead signal using the MACSPlex Exosome Kit in samples from patients with ovarian cancer because these patients are known to have increased levels of TF+ EVs (Figure 3). There was no correlation between EVTF activity and the TF bead signal in the samples ($r = -.24$; $P = .43$). In contrast, Balbi et al.[18] reported a correlation between EVTF activity, measured using the Tissue Factor Activity Assay (Abcam, Cambridge, UK), and the TF bead signal in serum samples from patients with COVID-19 ($r = .526$, $p < 0.001$).

There are several notable differences in the experimental design between our study and that of Balbi et al.[18] First, we isolated EVs isolated from plasma samples as recommended by the MACSPlex Exosome Kit to maximize the binding of EVs to beads instead of adding antibody-labeled beads directly to serum samples. Serum may contain factors that interfere with binding of antigen to antibodies. Second, we used plasma rather than serum because we found that plasma had much higher levels of EVTF activity compared to serum. Third, we used TF-positive and TF-negative controls prepared from whole blood from healthy donors and plasma samples from patients with ovarian cancer instead of only serum samples from healthy controls and patients with COVID-19. This allowed us to analyze samples with or without TF+ EVs. We believe that an assay for measuring TF antigen in EVs from plasma should be able to distinguish between TF-positive and TF-negative controls. Finally, we did not normalize the TF bead signal to the average of the 3 exosome markers CD9, CD63, and CD81 because we observed a large variation (CD9: 82-1362 MFI; CD63: 26-1063 MFI; CD81: 3-39 MFI) in the values among these three markers, and we did not think that this could be used to normalize bead signals to the number of EVs in the sample. It is unclear why Miltenyi Biotec recommend using the three markers to normalize different samples.

It is possible that our failure to detect TF antigen in EVs using the MACSPlex Exosome Kit is because TF is primarily carried on

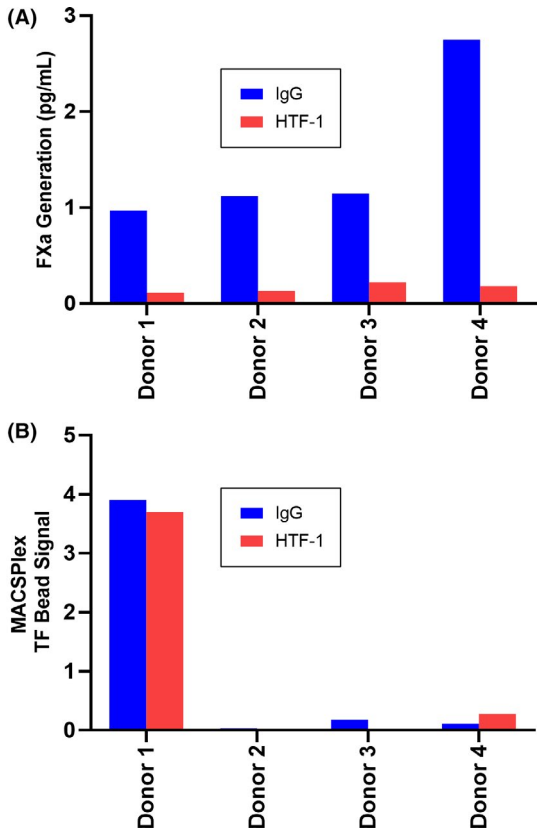

FIGURE 2 Effect of tissue factor (TF) inhibition on factor Xa (FXa) generation and tissue factor bead signal. Extracellular vesicles (EVs) were isolated from plasma from whole blood from four healthy donors with stimulation with lipopolysaccharide (LPS) with control antibody (IgG; blue bars) or anti-TF antibody HTF-1 (red bars) (final concentration: 7.8 μg/mL). (A) FXa generation of TF+ EVs in the presence of HTF-1 or IgG. (B) TF bead signal in EVs was measured using the MACSPlex Exosome Kit in the presence of HTF-1 or IgG (15 μg/mL) in EVs in plasma

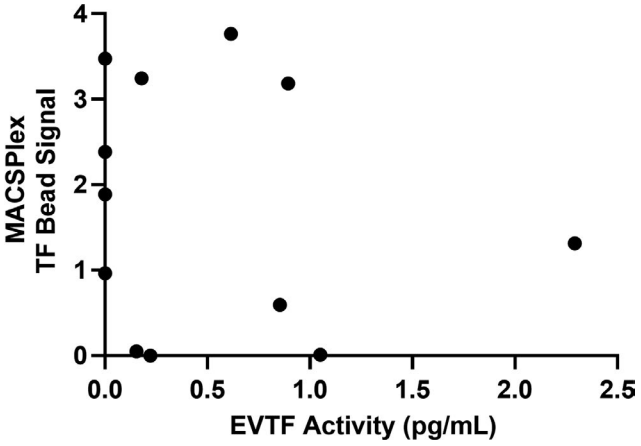

FIGURE 3 Levels of extracellular vesicle tissue factor (TF) activity and the TF bead signal in extracellular vesicles (EVs) isolated from plasma from patients with ovarian cancer. EVs were isolated from plasma from patients with ovarian cancer and EVTF activity and TF bead signal were measured using an in-house activity assay and the MACSPlex Exosome Kit, respectively ($r = -.24$; $P = .43$. Spearman's rank correlation coefficient, n = 13)

microvesicles rather than exosomes. We previously found that pelleting EVs using 20 000 *g* for 15 minutes yielded a similar amount of EVTF activity as using 100 000 *g* for 15 minutes.[11] This suggested that TF is present on larger microvesicles rather than smaller exosomes. However, centrifuging plasma at 20 000 *g* will pellet large EVs, residual platelets, and cellular debris. Unfortunately, the small amount of sample from patients precludes isolation of EVs using size exclusion chromatography columns. We also failed to detect a signal when we used an APC-labeled TF antibody as a detector.

Our studies indicate that the MACSPlex Exosome Kit does not detect TF+ EVs in plasma. This is most likely because the level of TF + EVs is below the threshold of detection of the assay. Indeed, the values for TF are >100 times lower than the signal for CD41b+ EVs. The MACSPlex Exosome Kit could not distinguish between TF-positive and TF-negative controls; the signal was not reduced by an anti-TF antibody; and the signal did not the correlate with EVTF activity in patients with ovarian cancer.

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RELATIONSHIP DISCLOSURE

The representatives from Miltenyi Biotec, Inc. measured bead signal using the MACSQuant Analyzer 10 flow cytometer in a blinded manner.

AUTHOR CONTRIBUTIONS

NM designed the study. SJA and YH performed experiments. VLB collected plasma samples from patients with ovarian cancer. NM wrote the manuscript. SJA and YH edited the manuscript. VLB approved the manuscript.

ORCID

Sierra J. Archibald https://orcid.org/0000-0002-6052-5680
Yohei Hisada https://orcid.org/0000-0001-9157-0524
Nigel Mackman https://orcid.org/0000-0002-9170-7700

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