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Microparticles as biomarkers of lung disease: enumeration in biological fluids using lipid bilayer microspheres

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Microparticles as biomarkers of lung disease: enumeration in biological fluids using lipid bilayer microspheres. Am J Physiol Lung Cell Mol Physiol 310: L802–L814, 2016. First published March 4, 2016; doi:10.1152/ajplung.00369.2015.—Extracellular vesicles, specifically microparticles (MPs), are rapidly gaining attention for their capacity to act as biomarkers for diagnosis, prognosis, or responsiveness to therapy in lung disease, in keeping with the concept of precision medicine. However, MP analysis by high-sensitivity flow cytometry (FCM) is complicated by a lack of accurate means for MP enumeration. To address this gap, we report here an enhanced FCM MP gating and enumeration technique based on the use of novel engineered lipid bilayer microspheres (LBMs). By comparison of LBM-based MP enumeration with conventional bead- or fluorescent-based FCM enumeration techniques and a gravimetric consumption gold standard, we found LBMs to be superior to commercial bead preparations, showing the smallest fixed bias and limits of agreement in Bland Altman analyses. LBMs had simultaneous capacity to aid FCM enumeration of MPs in plasma, BAL, and cell culture supernatants. LBM enumeration detected differences in MP counts in mice exposed to intraperitoneal lipopolysaccharide or saline. LBMs produced for 1) higher sensitivity for gating MPs populations, 2) reduced background within MP gates, 3) more appropriate size, and 4) an inexpensive alternative amenable to different fluorescent tags. LBM-based MP enumeration was useful for a series of different FCM systems assessed, whereas LBM gating benefited high- but not low-sensitivity FCM systems compared with fluorescence gating. By offering exclusive advantages over current means of gating and enumerating MPs, LBMs are uniquely suited to realizing the potential of MPs as biomarkers in biological lung fluids and facilitating precision medicine in lung disease.

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THE PROFOUND EFFECT OF LUNG DISEASE ON HUMAN LIFE is reflected by a tremendous socioeconomic burden exceeding 6.6 billion dollars annually in the UK, where pulmonary diseases account for a large percentage of emergency and inpatient visits and ultimately lead to one in five deaths in the UK (22). Chronic lung diseases can remain asymptomatic for a long time, often having progressed to an advanced stage by the time of diagnosis (12, 37, 51). At present there is a deficit of appropriate diagnostic tools to effectively detect lung disease at an early stage, to predict its progression and exacerbations, and to monitor the efficacy of treatment strategies (27). Science and medicine are rapidly evolving to a point to truly allow for precision medicine, central to which is the ability to detect specific signals (biomarkers) that are able to predict outcomes and monitor therapy (1, 23, 36). The ideal biomarker should have a high sensitivity and specificity for predicting clinical outcome or measuring recovery and be easy to sample, measure, and safely collect as well as be reproducible across multiple sites (17). Extracellular vesicles (EVs), specifically microparticles (MPs) represent emerging biomarkers that satisfy many of these ideal indices and may be quickly translatable to clinical practice. EVs define a spectrum of small lipid cell-derived vesicles with differing compositions and origins (reviewed in in Refs. 13, 21, and 30). In the present work, we focused specifically on microparticles (MPs), a subset of EVs of ~100–1,500 nm in size that are formed from externalization of inner lipids from the bilipid cell membrane during activation, stress, or apoptosis that are characteristically studded with membrane surface receptors and contain cargos originating from the parent cell that can remain functional in terms of cytokines, proteins, genetic material (micro/mRNA), lipids, or even organelles such as mitochondria (11).

Although EVs can be detected in nearly all biological fluids (45), MPs are particularly pertinent to the study of lung diseases, as the high blood flow and vascular surface area as well as easy access to airway- and airspace-derived samples create relevance to MPs in the blood, sputum, or bronchoalveolar lavage (BAL) fluid, something unparalleled by other organs (8, 30, 31, 34). Pioneering biomarker studies involving MPs in pulmonary conditions include diseases such as pulmonary hypertension (2, 3, 7, 32), lung cancer (44, 49), chronic obstructive pulmonary disease (COPD) (42, 43), and effects of smoking (15, 16, 19), which showcase MPs as abundant, accessible, and sensitive means of predicting disease while concomitantly hinting at their crucial role as active players in the pathomechanisms of lung disease and their capacity to act...
as shuttles to deliver therapeutics (30) that differentiate them from other canonical biomarkers. The ability to assess MP phenotypic origin, antigenic composition, and spatial-temporal profiles offers powerful diagnostic and prognostic information but at present desperately lacks accuracy and standardization. The most popular strategy for MP assessment is flow cytometry (FCM). Current FCM analyses, however, have major limitations pertaining to accurate MP gating (identification) and reproducible enumeration to accurately and simply measure MPs in biological fluids due to their small sizes, often approaching the limits of detection of FCM, as well as problems with standardization of measurements in different biological fluids, reproducibility over time, and across different machines. Accordingly, there is a growing need for reliable, standardized, user-friendly, sensitive, and accurate FCM enumeration of MPs that would enable meaningful comparison of MP findings between studies (40). Present attempts to address this problem include the use of both FCM gating and enumeration beads (4–6, 10, 24, 35, 40) or fluorescence strategies (28, 29, 33) with labels such as carboxy fluorescein succinimidyl ester (CFSE) (48) or annexin V (4, 24, 38, 39, 50). Each of these strategies, however, is hampered by major challenges in terms of preanalytical limitations, their sensitivity to detect MPs, their complexity, and suitability across different FCM platforms with differing capacities to measure MPs.

To address these present limitations, we aimed to devise a more accurate and standardized means of gating and enumerating MP measurements in pertinent pulmonary biological fluids that would be compatible with flow cytometers of differing abilities to gate and enumerate MPs. To address this gap, we have created biologically relevant lipid bilayer microspheres (LBMs) that are highly consistent in size (2 μm), containing phospholipids and proteins, and have FCM side (SSC) and forward scatter (FSC) characteristics, making them ideal for enumeration and upper gating markers for MP populations. Here, we show that LBMs offer clear advantages compared with current strategies in both gating and enumeration of MPs in different biological fluids (blood, BAL) via FCM analysis and thus present a major advancement for the reproducible and user-friendly assessment of MPs as a biomarker in lung disease.

METHODS

Biological fluids for MP enumeration. All animal experiments were approved by the St. Michael’s Hospital Animal Care Committee. Eight- to 10-wk-old male BALB/c mice (Charles River Laboratories) were housed with free access to water and standard mouse chow. Select mice were given single intraperitoneal (ip) injections of 2 mg/kg lipopolysaccharide from E. coli 0111:B4 (LPS; Sigma-Aldrich) or 0.9% normal saline (NS) as vehicle control 8 h prior to acquisition by FCM. The mice were anesthetized, and blood samples were collected via cardiac puncture after exsanguination and placed in 10 mL syringe containing 0.3 mL ethylenediaminetetraacetic acid (EDTA) as a blood anticoagulant. After centrifugation, the plasma fraction was separated, and supernatants were frozen at −80°C until time of analysis. Mouse plasma samples were diluted in sterile 200-nm filtered annexin V staining buffer (10 mMol/l HEPES, 140 mMol/l NaCl, and 2.5 mMol/l CaCl2, pH 7.4, in ddH2O; BioFlow, Burlington, ON, Canada) prior to being assessed by FCM.

Lipid bilayer microspheres. LBMs were suspended in sterile PBS at a known concentration (10.22 mg/mL) and dual leaflet composition of a physiological cell membrane. LBMs were grown as 32 × 106 cells/flask and passage once in serum-free media containing phospholipids and proteins, and have FCM side (SSC) and forward scatter (FSC) characteristics, making them ideal for enumeration and upper gating markers for MP populations. Here, we show that LBMs offer clear advantages compared with current strategies in both gating and enumeration of MPs in different biological fluids (blood, BAL) via FCM analysis and thus present a major advancement for the reproducible and user-friendly assessment of MPs as a biomarker in lung disease.
Flow cytometric microparticle gating. Establishing a window of accurate observation for counting and characterizing MPs by FCM involved biexponential forward and side scatter plots in conjunction with either light scatter- (small bead gate or large LBM gate) or fluorescence-based (fluorescence gate) gating (Fig. 1).

Light scatter MP gating involved the use of two different strategies. First, certain MPs containing samples were gated by small-particle calibration gating beads (polystyrene microspheres; 300, 500, 800, and 1,000 nm in size; BD Biosciences), where the 300- and 800-nm beads established the lower and upper gating limits, respectively (small bead gate; Figs. 1A, 2A, C, 3B, C, and 9B). Alternatively, MP-containing samples were gated by light scatter based on excluding debris/instrument noise on the lower limit and LBM gating particles (2,000 nm in size) as the upper boundary (large LBM gate; Figs. 1B, 2B, D, 3B, D, 4, 5, 6, 7, 8, and 9A).

For fluorescence-based MP gating, MP events were identified by fluorescence positivity following MP staining by 5 μMol/l CFSE (Life Technologies, Burlington, ON, Canada) (fluorescence gate; Fig. 1C). Background contribution from gating aids such as beads and LBMs showed a higher rate of false-positive events in the small bead gate compared with the large LBM gate (Fig. 1A and B), giving a first indication of the superiority of LBMs for conventional bead-based approaches.

For select experiments, MPs were labeled by annexin V conjugated to phycoerythrin (BD Biosciences) 10 min prior to FCM analysis. Appropriate controls containing 5 mmol/l of the Ca2+ chelator EDTA (Sigma-Aldrich) were performed, which eliminated fluorescent events within the MP gates, in line with the requirement annexin V labeling for Ca2+ (data not shown).

Flow cytometric enumeration of microparticles. FCM enumeration of MP/μl sampled from diluted BAL, plasma, and cell culture supernatants was obtained by either 1) gravimetric consumption during sample uptake, 2) enumeration of known spiked concentrations of LBMs, or 3) enumeration by popular commercial counting beads (rainbow and ultrarainbow beads; Spherotech, Lakeforest, IL), Fluoresbrite YG carboxylate microspheres (Polysciences beads, 500 nm; Polysciences, Warrington, PA), and Trucount beads (BD Biosciences).

Gravimetric consumption during FCM measurement took into account the specific gravity of the diluted biological sample being assessed, namely 1.0095 for BAL, 1.014 for plasma, and 1.006 for cell culture supernatant. The concentration of MPs (MPs/μl) was then calculated as

\[
MP/\mu l = \frac{\text{MP count from LBM gate}}{\text{LBM spiked in sample/\mu l} \times \text{specific gravity in 1 ml}}
\]

Alternatively, known concentrations of LBMs were spiked in diluted biological fluid samples to allow for calculation of MP concentration as

\[
MP/\mu l = \frac{\text{no. of MP counted from LBM gate} \times \text{LBM spiked in sample/\mu l}}{\text{no. of LBM counted} \times 1,000 \mu l/\text{ml}}
\]

Fig. 1. Flow cytometric microparticle (MP) gating strategies. Representative images show forward (x-axes in A and B) vs. side scatter (y-axes in A–C) plots from flow cytometric analyses of either polystyrene beads, lipid bilayer microspheres (LBMs), or carboxy fluorescein succinimidyl ester (CFSE)-labeled MP fluorescence (x-axis in C) in sterile, filtered annexin V staining buffer (A–C, left). A: MP gating based on light scatter of polystyrene beads (small bead gate) bounded by 1,000-(top right corner outside the bead gate; green), 800- (top right corner inside the bead gate), 500- (middle of the bead gate), and 300-nm (bottom left corner inside of the bead gate) beads (purple) in the absence of actual MPs. Note the bead debris (under the bead gate; blue) and bead doublets (top right corner outside the bead gate; black), as well as instrument noise (bottom; black). B: MP gating based on light scatter of LBMs (top right corner outside the MP gate; red) in the absence of actual MPs with some LBMs debris (blue events) within the LBM-to-noise MP gate (large LBM gate). C: MP gating by use of CFSE fluorescently labeled MPs (fluorescence gate) from mouse bronchoalveolar lavage fluid (green). Note the small nonfluorescent MPs (left of MP gate; blue), fluorescently labeled LBMs (right of MP gate; red), and instrument noise (bottom left; black). In each flow cytometry plot (left), the actual MP gate is shown delimited by thick black borders. To emphasize the differences in size of light scatter MP gates, we show both the small bead gate (small box) and large LBM gate (large box) superimposed together (A). To exclude MP events, 0.05% Triton X-100 solubility of MP samples was determined (A–C, right). Shown are MP counts of diluted mouse bronchoalveolar lavage fluid given as 100% (black bars) and the residual MP counts after treatment with 0.05% Triton X-100 (gray bars) on the ARIA III cytometer for gating based on the small bead gate (right bar graph; A), large LBM gate (right bar graph; B), and fluorescence gate (right bar graph; C). Residual MP counts on bar graphs post-Triton X-100 data are given as means ± SD; n = 3 each.
Finally, known concentrations of four different popular commercially obtained counting beads were used for enumeration; rainbow and ultrarainbow beads, Fluoresbrite YG carboxylate microsphere beads (Polysciences beads; 500 nm), and Trucount beads were spiked in diluted biological fluid samples, and MP concentration was calculated as:

$$\text{MP/\muL} = \left( \frac{\text{(no. of MP counted from bead gate)}}{\text{(no. of enumeration beads counted)}} \right) \times \left( \frac{1,000 \text{ \muL/mL}}{\text{no. of enumeration beads spiked in sample/mL}} \right)$$

MP counts were generated by multiplexed analyses from the same tube(s) to eliminate intersample variability when comparing enumeration strategies.

Calibration controls for flow cytometric assessment of MPs. When assaying MPs in biological fluids, appropriate controls are essential to ensure extracellular vesicles are being measured and not similarly sized aggregates, multiple events, or debris. To test the contribution of non-MP events such as immune complexes or debris with light scatter-based gating, we selectively depleted MPs by the addition of 0.05% Triton X-100 (Sigma-Aldrich) to samples. Triton X-100 treatment reduced MP counts for all gating strategies assessed with all flow cytometers used in the study by >87% (for ARIA III, see Fig. 1; other data not shown), confirming that non-MP events did not contribute significantly to MP gated counts. Serial dilutions (1:10, 1:100, and 1:1,000) were performed to assess coincidence (“swarm detection”, i.e., measurement of MP aggregates or multiple MPs passing the detector at the same time). There was no change in MP counts across dilutions in biological fluids an order of magnitude above and below the standard utilized dilution (data not shown).

**Statistical analysis.** All data are given as means ± SD. Comparisons between groups were performed by two-tailed unpaired Student’s t-test ($P < 0.05$) or two-way analysis of variance ($P < 0.05$). Different enumeration strategies were compared by linear regression analysis, correlation coefficient, and Bland-Altman analysis (9). All statistical tests were completed with Prism software 5.00 (Graphpad Software).

**RESULTS**

Different gating and enumeration strategies impact microparticle counts in biological fluids. In a first set of experiments, we compared different MP gating and enumeration methods against gravimetric counting, which served as a gold standard, within a single sample of BAL, as it is a commonly used biological fluid central to the study of lung disease. When using the small bead gate (Fig. 2C) or the large LBM gate (Fig. 2D) within the same diluted BAL sample, enumeration by beads was less accurate in counting total events compared with gravimetric MP estimates, but not ultrarainbow beads and gravimetric counts (due to the large variability of ultrarainbow bead MP estimates), of all the techniques examined the LBMs most closely approximated gold standard gravimetric counts.
Specifically, counting beads either undercounted (rainbow beads) or overcounted (ultrarainbow beads) compared with gravimetric counts, regardless of whether the large or small light scatter based gates were used. Next, we compared these light scatter gating and enumeration techniques (small bead and large LBM gates) with CFSE fluorescence gating. Fluorescence gating showed a tendency to enumerate more MPs compared with the light scatter based small bead gate for all three enumeration strategies; this trend reached significance in the case of the LBM-based enumeration (Fig. 3A). Conversely, fluorescence gating tended to count fewer MPs compared with light scatter-based large LBM gates, which again reached significance for LBM-based enumeration (Fig. 3B).

Having thus established that the most effective gating method using high-sensitivity FCM was large LBM gates, we next compared different enumeration strategies in different biological fluids (BAL, plasma, and cell culture supernatants) with respect to their accuracy and reproducibility over a range of different MP concentrations. To this end, we applied the large LBM gate strategy that had provided the largest window for MP detection in BAL and compared gravimetric, LBM-based, and popular commercial enumeration bead strategies by Blad Altman analysis (9). Because of their tendency to significantly overcount MPs, ultrarainbow enumeration beads were not pursued in subsequent analyses. In BAL fluid, strong linear correlation with gravimetric consumption was evident for all tested enumeration strategies, i.e., Rainbow beads ($r^2 = 0.99$), LBMGs ($r^2 = 0.99$), Polysciences beads ($r^2 = 0.96$), and Trucount beads ($r^2 = 0.93$). However, Bland Altman analysis revealed distinct differences between these four strategies (Fig. 4). Bland Altman plots display the difference between the measured results of two methods (a tested method and a gold standard; gravimetric counting) against the average of both methods. The mean difference (gravimetric counts, counts from tested method) on the y-axis was closest to 0 for LBMs, which indicates the least-fixed bias of the four techniques compared with the gravimetric results. Furthermore, LBMs had the smallest limits of agreement (average difference ± 2 standard deviations on the y-axis), indicating good reproducibility within BAL fluid across the range of concentrations assessed. In contrast, all three bead-based techniques showed relatively large limits of agreement and a concentration-dependent bias, visible as an upward trend in measured values as the MP concentration increased, indicating an increasing inability
to accurately count MPs at higher concentrations (Fig. 4A). Next, we tested the enumeration capacity of the above methods in plasma (Fig. 5). The correlations between gravimetric consumption and LBMs ($r^2 = 0.83$), Rainbow beads ($r^2 = 0.51$), Polysciences beads ($r^2 = 0.66$), and Trucount beads ($r^2 = 0.66$) were again high, albeit overall less so than for BAL. However, in plasma, both LBMs and beads had wider limits of agreement than in BAL, indicating greater variability across different concentrations. LBMs showed the least-fixed bias, yet limits of agreement for Polysciences beads were almost twice as large as for LBMs. Of the four methods tested, Trucount beads had the narrowest limits of agreement in plasma, yet their fixed bias was greater than that of LBMs. In cultured cell supernatants (Fig. 6), there was again a good correlation between gravimetric consumption and LBMs ($r^2 = 0.74$), Rainbow beads ($r^2 = 0.96$), Polysciences beads ($r^2 = 0.70$) and Trucount beads ($r^2 = 0.71$). LBMs performed best in terms of fixed bias and limits of agreement, closely followed by Polysciences beads and markedly superior to Rainbow and Trucount beads (Fig. 4). Taken together, these quantitative comparisons reveal that the various enumeration strategies assessed with the large LBM gate vary in their accuracy between each other as well as within the same enumeration strategy when used in different biological fluids. Notably, LBMs had the least-fixed bias within all fluids examined and the smallest limits of agreement, except for plasma, compared with all three bead-based strategies.

Based on these data, we next probed the ability of LBMs for gating and enumeration of MPs in a preclinical model of murine lung inflammation. To this end, we assessed MP levels in both BAL and plasma from mice treated 8 h prior to sampling with ip injections of either NS or 2 mg/kg body wt LPS. By use of the large LBM gate and enumeration, we detected a marked increase in plasma MP counts in LPS- vs. NS-treated mice, whereas MP counts remained unchanged within BAL (Fig. 7). Hence, LBM gating and enumeration successfully allowed for semiquantitative assessment of differences in circulating MPs numbers in healthy vs. inflamed mice.

LBMs assist MP gating and enumeration with different flow cytometers and configurations. High sensitivity FCM with the large LBM gate and LBMBased enumeration detects biologically relevant differences in MP counts with a high degree of

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**Fig. 4.** Bland-Altman analyses of MP counts as determined by different light scatter-based strategies in murine bronchoalveolar fluid (BAL). MP counts were determined by LBM light scatter-based large LBM gates and enumeration by LBMs (A) or 3 different bead-based enumeration methods, namely Rainbow beads (B), Polysciences 500-nm beads (C), and Trucount beads (D) each in relation to MP counts determined in the same sampling tubes by gravimetric consumption as gold standard. Bland-Altman plots depict for each method the difference between the determined counts and the counts measured by the gold standard against the mean count from both techniques. Bland-Altman analyses revealed the least-fixed bias (deviation of the mean from 0 on the y-axis; dotted line) and smallest limits of agreement (means ± 2 SD on the y-axis; dashed lines) for LBMs compared with all bead-based techniques, which in addition showed a consistent concentration-dependent bias. Data are shown as individual data points from $n = 20$ different measurements for each comparison.
precision in different biological fluids. Yet to establish the
general utility of LBMs for measuring MPs, it is critical to
assess how well LBMs perform in different FCM systems with
differing capacity to measure MPs. To this end, we compared
the ability of different flow cytometers to detect MPs with
LBMs. Within the chosen FCM systems, we compared the top
two gating strategies tested consisting of both light scatter
gating with the large LBM gate and fluorescence gating with
CFSE (fluorescence gate) to assess which of these strategies
would be most advantageous on each machine for LBM-
assisted enumeration. Furthermore, we compared the large
LBM gate to annexin V fluorescence gating due to its use in
previous studies as a means of gating MPs.

Of the cytometers tested with LBM based gating, the ARIA
III had the highest MP detection sensitivity in BAL samples,
followed by the Cytoflex and X-20, which had higher counts
than the Calibur and MacsQuant (Fig. 8A). Similar trends in
sensitivity were seen with CFSE fluorescence gating. Unlike
more sensitive FCM systems where the large LBM gate yielded
higher MP counts than CFSE fluorescence gating, less
sensitive machines such as the Calibur and the MacsQuant
yielded higher MP counts with the fluorescence rather than the
large LBM gate. Therefore, fluorescence gating on lower-
resolution cytometers allows for fluorescent discrimination of
smaller MPs that would otherwise be lost in the instrument
noise on light scatter gates, whereas more sensitive machines
detect more MPs with the large LBM gate compared with the
fluorescence gate. We considered that the differences in the
abilities of these five instruments to resolve MPs may relate at
least in part to their differences in laser power. We thus tested
the effect of power of the 488-nm laser within the X-20 on the
ability to quantitatively detect MPs by use of the large LBM
gate. With decreasing laser power the light scatter sensitivity
for detection of MPs decreased linearly (Fig. 8B), indicating
that differences in laser power may contribute markedly to the
varying abilities of different cytometers to accurately resolve
and enumerate MPs.

Finally, we tested whether gating and enumeration could
affect the visibility of annexin V/H11001 MPs, as annexin V positivity
has historically been used as marker for MPs. To this end, we
compared an annexin V fluorescence gate to the large LBM
gate in BAL samples over a range of cytometers. Similarly to
CFSE gating, annexin V staining showed higher or at least
equivalent MP counts compared with light scatter-based large
LBM gates for low-sensitivity machines (Calibur, MacsQuant),
whereas high-sensitivity instruments (ARIA III, Cytoflex,
Lipid bilayer microspheres matched with high-sensitivity FCM are capable of more accurately simultaneously gating and enumerating MPs compared with combinations of popular commercial enumeration and gating beads or fluorescence gating combined with LBM enumeration. LBM-based FCM analysis was able to effectively detect elevated MPs between sham- and LPS-treated mouse plasmas. LBMs offer gating advantages for high-sensitivity FCM and an effective enumeration aid for all FCM systems assessed.

The present investigation was driven by the unmet need for more accurate, user-friendly, and cost-effective ways to identify and enumerate MPs in biological samples relevant to lung disease. The characterization of MPs is challenging due to their small size, and there currently exists no gold standard for the detection and analysis of MPs (46). FCM was chosen in the present study as the means of enumerating MPs because it enjoys the greatest popularity among MP investigators, as it is commonly accessible and able to count large sample numbers and phenotype subpopulations with the use of fluorescently tagged antibodies. FCM, however, is hampered by an inability to discriminate the exact size of biological vesicles and their shape and fundamentally lacks the appropriate reference materials necessary to standardize MP enumeration effectively (24, 28, 48). Therefore, we aimed to resolve the crucial gap of accurate MP gating and enumeration strategies by use of a novel light scatter strategy that is based on the use of small lipid bilayer microspheres as a reference. Here, we demonstrate that LBMs enhance high-sensitivity FCM for both gating and enumeration of MPs and also benefit older cytometers with limited MP detection capabilities.

FCM enumeration of MPs is challenging, as MPs are frequently smaller than the wavelength of incident laser light and thus invariably cross into background noise at the lower detection limits of cytometers. To overcome limitations with current gating strategies of light scatter bead-based gating (4–6, 10, 24, 35, 40) or CFSE/annexin V fluorescence gating (4, 24, 38, 39, 41, 48, 50), we designed a gating strategy based on the use of small lipid bilayer microspheres as a reference. Here, we demonstrate that LBMs enhance high-sensitivity FCM for both gating and enumeration of MPs and also benefit older cytometers with limited MP detection capabilities.

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the upper size limit characteristic for MPs (100-1,000 nm). LBMs resemble MPs in their composition of phospholipids and proteins, which is important since refractive indexes, shapes, material composition, and Mie and Rayleigh light scatter are critical in enumerative comparisons (reviewed in Ref. 46).

Fig. 7. Compartmental analysis of MP generation in systemically inflamed mice. MP counts in BAL and plasma from mice that had received an intraperitoneal challenge with either normal saline (NS; black bars) or 2 mg/kg lipopolysaccharide (LPS; gray bars) from E. coli 0111:B4 8 h prior to sample collection were determined with novel LBM light scatter-based large LBM gates and LBM-derived enumeration. Significantly elevated numbers of MPs were detected in plasma samples of LPS-treated compared with NS-treated mice, but not in BAL samples. Data are given as means ± SD; n = 3. *P < 0.05 vs. NS plasma.

Light scatter-based large LBM gates using high-sensitivity FCM facilitated a larger gate for MP observations than commercial beads or fluorescence strategies. This was in part due to measuring down to just above instrument noise at the low end, which expanded the window of observation to capture MP events. This is significant because it allows a disproportionately greater fraction of extracellular vesicles in biological fluids to be measured, which are typically skewed to smaller sized MPs (<300 nm) (4, 5, 20, 46, 47). An additional advantage of LBMs is their slightly larger size compared with MPs, which positions them just outside the MP gate as opposed to inside, as seen with most commercial beads, which can add complexity to interpreting MP observations in bead-derived MP gates. Beads are further hindered relative to LBM gating by the fact that different FCM machines resolve beads differently or simply cannot resolve smaller beads adequately (14). Unlike LBMs, beads are costly as they are either usually bought as a cocktail of different-sized beads or individually to create a custom range of sizes to suit the investigators’ needs. Separate gating and counting of beads adds to complexity and cost, unlike the economical duality of LBMs, which serves both tasks simultaneously. It should be noted, however, that despite the demonstrated advantages of LBMs over bead-based strategies, instrument noise defining the lower limit of large LBM gates remains the final hurdle of fully standardized light scatter gated MP enumeration. Instrument noise at the lower MP gate varies between machines, and over time this limits true MP enumeration at present to relative observations between sample groups with LBMs. Although relative counts of

Fig. 8. MP enumeration by different flow cytometers using light scatter- vs. fluorescence-based gating strategies. A: MP counts in murine BAL were determined among different flow cytometers with differing capacities to gate and enumerate MPs. Analyses were performed on identical sample tubes that were enumerated in a multiplexed manner to reduce intersample variability. Gating was performed with either LBM-based light scatter gates (large LBM gate; black bars) or fluorescent CFSE+ gating (fluorescence gate; gray bars), whereas counting estimates involved enumeration with LBMs. Within individual cytometers, marked differences between light scatter and fluorescence gating were noticeable, with instruments with high-sensitivity (ARIA III, X-20, Cytoflex) tending to undercount and instruments with lower sensitivity (Calibur) tending to overcount with fluorescence gating compared with light scatter gating. MP estimates also varied considerably between different cytometers. Data are given as means ± SD; n = 5. *P < 0.05. B: to assess the influence of different laser powers on this variability, additional MP counts in BAL were determined on a BD X-20 SORP cytometer with light scatter gating using the large LBM gate- and LBM-based enumeration, whereas the forward scatter laser power was titrated to 20, 25, 50, 75, and 100 mW. MP counts correlated near-linearly with laser power (r² = 0.97, P < 0.05).
Healthy vs. disease states can easily be accomplished, as evidenced by the detected changes in MPs in LPS-treated mice, multicenter and temporally spaced trials are currently hampered by a lack of reference material to define the lower MP gating boundaries.

Although conventional cytometers assisted by fluorescence gating continue to evolve with better low-end detection limits to allow detection of >100-nm MP-sized vesicles (4, 5, 48), in the present study the ARIA III was able to detect even more MPs by the large LBM gate than with fluorescence. Thus a balance exists in which fluorescence-based discrimination provides an advantage if the cytometer in question has significant light scatter resolution limitations, whereas cytometers with superior light scatter sensitivity operate even more effectively without fluorescence discrimination. The ability to detect and enumerate MPs without the use of fluorescence gating may prove particularly useful as 1) it avoids potential measurement error due to nonspecific uptake of fluorescence, 2) it reserves fluorescence channels for phenotypic investigations by key bright fluorophores, 3) it evades false-negative or false-positive results due to poor uptake of fluorescent markers in smaller MPs or formation of fluorescent protein aggregates, including labeling of serum proteins, respectively, and 4) it eliminates problems arising from background fluorescence and the difficulties to washout unbound dye. Like CFSE, annexin V fluorescent gating was advantageous for increasing MP numbers counted with low- but not high-sensitivity FCM. Although annexin V continues to enjoy some popularity as a means of identifying MPs by fluorescence (4), a growing body of data demonstrate that annexin V is not present in all MPs (40). Although assessment of annexin V positivity remains of interest in the study of lung pathology due to its association with phosphatidylserine, which has procoagulant properties (18, 26), its usefulness for MP enumeration is limited and inferior to light scatter-based techniques when high-sensitivity FCMs are applied.

Although LBMs and the various bead-based techniques correlated well with gold standard gravimetric counts, there were important divergences in enumeration capabilities when stratified by Bland Altman analyses. In all biological solutions examined, LBMs offered significant advantages for several reasons. An immediate advantage is the shared capacity to both gate and enumerate simultaneously, which reduces cost and complexity, as commercially available beads are typically purchased separately for enumeration or gating. As an enumeration method for MPs in BAL and cell culture supernatant, LBMs were superior to popular counting beads due to their low fixed bias, smaller limits of agreement, and lack of concentration-dependent bias. However, LBMs’ advantages were less prominent in plasma, where they were superior in terms of

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**Fig. 9.** Comparison between light scatter gating and enumeration techniques vs. fluorescence gating by annexin V positivity. MP counts in murine BAL were determined on different cytometers with LBM light scatter-based (large LBM gate; A) or small bead gate- (B) and LBM-based enumeration (black bars) or Annexin V+ fluorescence events (gray bars) enumerated by LBMs. MP counts were significantly higher for light scatter compared with annexin V positivity within the same samples examined by the same cytometer for instruments with high sensitivity (ARIA III, X-20) yet lower in instruments with lower sensitivity (Calibur). Light scatter MP counts were significantly higher with the large LBM gates compared with annexin V-positive counts using an ARIA III yet were significantly lower than annexin V-positive counts when gating with smaller bead based gates. Data are depicted as means ± SD; n = 5. *P < 0.05.
fixed bias but slightly inferior in terms of limits of agreement compared with Rainbow beads and Trucount beads, respectively. Unlike LBMs, bead-based enumeration strategies have additional limitations that vary in severity among the different beads available and in different biological fluids. Beads are plagued with potential high background particulate contamination within MP gates (exemplified in Fig. 1A), which can lead to overcounting (ultrarainbow beads), possible adherence of beads within the cytometer tubing, large-sized beads that may settle in solution and overestimate the volume analyzed, leading to undercounting MPs (rainbow beads), or beads sized so they are found in MP gates (Polysciences beads), which complicates analysis. Across all biological fluids analyzed, LBMs offered the closest MP counts to the gold standard and offered greater simplicity and fewer false-positive events than beads.

Different biological fluids add challenges to MP enumeration due to their composition, viscosity, cellular endowments, and interactions with FCM systems such as intrinsic autofluorescence differences. In the present study, biological fluids were examined, ranging from the more aqueous low-background serum-free cell culture supernatant to the intermediate BAL and finally, more viscous and protein-rich plasma. Not surprisingly, given the potential for false-positive events due to protein aggregates, plasma proved the most challenging fluid with the widest limits of agreement for all tested MP enumeration strategies, even when taking into account the fact that on average higher MP concentrations were detected in plasma compared with other fluids, which in case of a concentration-dependent bias may contribute to the wider limits of agreement (e.g., in the case of Rainbow beads).

In order for MPs to be meaningful biomarkers, means of comparing and interpreting MP counts among different investigators using different FCM systems is critical (25, 40). Few studies so far have addressed potential differences between cytometers regarding enumeration of MPs (14, 24, 25, 35). LBMs will not resolve variability in MP detection between different FCM systems, which is attributable to hardware-specific limits of detection and signal noise. Notwithstanding, we show here that current high-sensitivity FCMs equipped with small-particle detection units clearly benefit from LBMs for gating and enumeration, whereas less sensitive instruments did not always benefit from LBMs gating, presumably because of a high-light scatter background contribution and lack of adequate sensitivity. Some of these limitations appear to be related to laser power, because when the X-20 laser was modulated from 100 down to 20 mW there was a linear relationship with the number of MPs counted. This relationship of laser power and MP counts was roughly reproduced among the different FCM systems examined with 488-nm laser powers ranging from 130 mW for the ARIA III to 25 and 15 mW in case of the MacsQuant and Calibur, respectively. MP enumeration by low-light scatter sensitivity FCMs using the large LBM gate yielded similar or even lower MP counts than CFSE or annexin V fluorescence counts. Hence, unlike high-sensitivity FCY, which clearly benefits from both LBM gating and enumeration, in FCM with poor light scatter resolution, CFSE fluorescence may help measure “dim” fluorescently positive small vesicles that would otherwise be lost in light scatter background noise (33, 48). Notably, LBMs still offer the advantage of a means of enumerating MPs within these machines.

Taken together, we report an accurate, reliable, and user-friendly technique for simultaneous light scatter MP gating and enumeration by use of LBMs that is overall superior to conventional bead or fluorescence-based strategies for FCM systems of differing abilities to detect MPs. LBMs gating and enumeration detected differences in MPs in different biological compartments in an LPS mouse model, showing their ability to facilitate MP biomarker measurements. In the present study, we focused the use of LBMs on gating and enumeration of MPs in biological fluids pertinent to lung disease. In consideration of the improved performance of LBMs compared with conventional enumeration strategies in different biological fluids, which varied in cellularity and viscosity, it seems fair to speculate that LBMs will likely perform equally well in non-pulmonary settings pertinent to health and disease. As we enter the dawn of precision medicine, MPs are poised to be a key player in driving this next evolution in pulmonary care. LBMs offer a novel powerful means of enhancing our ability to meaningfully determine semiquantitative differences in MPs over time and across different platforms, which in turn begins to unlock their potential as quantitative biomarkers of lung injury or mediators of therapeutic treatment of lung disease.

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Author Contributions

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