Heterogeneity in Neutrophil Microparticles Reveals Distinct Proteome and Functional Properties

Jesmond Dalli‡‡‡, Trinidad Montero-Melendez‡‡‡, Lucy V Norling‡‡, Xiaoike Yin§, Charles Hinds‡¶, Dorian Haskard¶¶, Manuel Mayr§, and Mauro Perretti‡**

Altered plasma neutrophil microparticle levels have recently been implicated in a number of vascular and inflammatory diseases, yet our understanding of their actions is very limited. Herein, we investigate the proteome of neutrophil microparticles in order to shed light on their biological actions. Stimulation of human neutrophils, either in suspension or adherent to an endothelial monolayer, led to the production of microparticles containing >400 distinct proteins with only 223 being shared by the two subsets. For instance, postadherent microparticles were enriched in alpha-2 macroglobulin and ceruloplasmin, whereas microparticles produced by neutrophils in suspension were abundant in heat shock 70 kDa protein 1. Annexin A1 and lactotransferrin were expressed in both microparticle subsets. We next determined relative abundance of these proteins in three types of human microparticle samples: healthy volunteer plasma, plasma of septic patients and skin blister exudates finding that these proteins were differentially expressed on neutrophil microparticles from these samples reflecting in part the expression profiles we found in vitro. Functional assessment of the neutrophil microparticles subsets demonstrated that in response to direct stimulation neutrophil microparticles produced reactive oxygen species and leukotriene B4 as well as locomoted toward a chemotactic gradient. Finally, we investigated the actions of the two neutrophil microparticles subsets described herein on target cell responses. Microarray analysis with human primary endothelial cells incubated with either microparticle subset revealed a discrete modulation of endothelial gene expression profile. These findings demonstrate that neutrophil microparticles are heterogeneous and can deliver packaged information propagating the activation status of the parent cell, potentially exerting novel and fundamental roles both under homeostatic and disease conditions. Molecular & Cellular Proteomics 12: 10.1074/mcp.M113.028589, 2205–2219, 2013.

The emerging notion that cells can communicate by packaged information represents a major shift in our understanding of cell-to-cell interaction in complex settings including inflammation (1). Packaging of mediators (irrespective of their chemical nature) in structures that can be transported through the vascular and lymphatic systems might avoid their rapid dilution and removal by biological fluids and allow the target cell or tissue to receive a biologically relevant amount of a given molecule. As an example, TNF-α produced by mast cells in the mouse paw can reach the lymph nodes unmodified, wrapped up in small structures or vesicles (2). In this respect, the last few years have witnessed augmented understanding in microparticle function.

Described over 50 years ago (reviewed in (3, 4), microparticles are heterogeneous in nature with their size varying between 0.2 and 1.0 μm, and are characterized by an outer membrane composed of a phospholipid bilayer and cell surface proteins. The mechanism of microparticle production is not fully understood, though it may follow processes not dissimilar from those observed in apoptosis, involving membrane detachment from the anchoring cytoskeleton and loss of membrane symmetry, which leads to exposure of negatively charged phospholipids (5–7). Proteins found on the outer leaflet of the microparticle cell membrane are believed to reflect both the origin and activation status of the parental cell (8, 9); for instance, microparticles from neutrophils express CD66b and CD62L (10, 11). We have recently identified the selective expression of the potent anti-inflammatory and proresolving protein Annexin A1 (ANXA1) on the surface of microparticles generated from neutrophils adherent to endothelial monolayers, when compared with those prepared from quiescent neutrophils (12). Microparticle production is not restricted to one subset of cells and using cell specific antigens the relative contribution of different cell types to the total microparticle population in a particular environment can be assessed. This has allowed for the analysis of different micro-
Heterogeneity in Neutrophil Microparticles

Microparticle populations (the focus being by and large platelet- and endothelial-derived microparticles) in a number of pathologies in the quest to identify robust biomarkers for disease and treatment (13–15). With regard to inflammatory diseases, examples would include plasma samples in sepsis (16), psoriatic arthritis (17), and scleroderma (18). However, the vast majority of these studies have only determined microparticle expression patterns with respect to the cell type of origin, without addressing the possibility that microparticle composition—even when generated from the same leukocyte subset—might differ in relation to disease status and/or mode of cell activation. Of note recent work has also demonstrated that the production of neutrophil microparticles during self-limited inflammation is temporally regulated suggesting that these microparticles are important in orchestrating inflammation-resolution (1).

Recent work has established that microparticles can elicit a variety of biological processes ranging from angiogenesis to anti-inflammation; so that it is very unlikely they can continue to be considered “cell debris,” as initially postulated. The following are some examples, relevant to the present study. Ingestion of platelet microparticles alters the phenotype of macrophages, leading to the false identification of endothelial cell progenitor cells in culture (19). Likewise, sonic hedgehog can be transferred, via microparticles, to dysfunctional endothelial cells, restoring the activity of nitric oxide synthase with downstream production of nitric oxide (20). Microparticles can carry functionally active receptor proteins to target cells (21, 22). Finally, in vivo generation of microparticles has been observed within the inflamed microcirculation. Real time analysis of leukocyte recruitment has visualized microparticle release from leukocytes squeezing through an endothelial barrier, providing evidence for their formation in vivo together with potential functional relevance in relation to cell migration (23).

On stimulation, neutrophils produce microparticles with rapid and nongenomic anti-inflammatory properties, in vitro and in vivo, reliant on their expression of ANXA1 (12). Whereas these findings are consistent with those obtained by Gasser and colleagues (24) who described inhibitory properties of neutrophil microparticles, other studies have suggested that the same cell type can produce microparticles that elicit activating properties, for instance upon incubation with endothelial cells or monocytes for longer time-points (25, 26). Thus, to gain further insight into the potential mechanisms involved in mediating such distinct effects, we deemed it important to determine the total proteome of neutrophil microparticles. Having established that different stimulation conditions yield microparticle populations with distinct protein profiles, we corroborated our observations in two distinct clinical scenarios, characterizing neutrophil microparticles from skin blister exudates and plasma samples from sepsis patients using a select group of proteins identified in our proteomic profile. We also established that the two microparticles subsets differentially modulate endothelial cell gene expression profile and thereby function, as determined by connectivity map analysis.

EXPERIMENTAL PROCEDURES

Unless otherwise specified, materials were obtained from Sigma-Aldrich Ltd (Poole, UK). Human cells were prepared according to a protocol approved by the local Research Ethics Committee (P/00/029 ELCHA). Plasma samples were obtained from patients suffering from severe sepsis/septic shock (defined according to The American College of Chest Physicians/Society of Critical Care Medicine consensus definitions) caused by community-acquired pneumonia (CAP) using a protocol approved by the Multicenter Research Ethics Committee (08/H0505/78).

Microparticle Generation and Characterization

Generation of Different Samples of Microparticles—Human neutrophil microparticles were prepared from peripheral blood neutrophils obtained from healthy volunteers as previously described (12). Two methods of neutrophil microparticle production were employed. In the first case neutrophils were resuspended in RPMI 1640 (Invitrogen, Paisley, UK) at a concentration of 2 × 107 cells/ml incubated for 20 min at 37 °C and then stimulated for a further 20 min at 37 °C with 1 μM fMLF: this subset of microparticles will be referred to, hereafter, as Fluid Phase or FIP1 microparticles. The second protocol involved pre-incubation of neutrophils, resuspended at 2 × 107 cells/ml, over a HUVEC monolayer for 20 min at 37 °C before addition of fMLF (1 μM; 20 min at 37 °C); this subset of microparticles will be referred to as Immobilized Phase or ImP microparticles. All incubation assays were conducted in the absence of fetal calf serum and, for the ImP microparticles. HUVEC monolayers were washed with PBS before addition of the neutrophils. In both cases, cell supernatants were collected and cells removed by two successive centrifugations at 3000 × g for 10 min at 4 °C, before pelleting the microparticles by centrifugation at 100,000 × g for 1 h at 4 °C, as described (12). Microparticle pellets were washed with Dulbecco phosphate buffered solution (DPBS), resuspended and stored at −80 °C before further analysis.

Blood (4 ml) from healthy volunteers or septic patients was centrifuged at 4 °C for 10 min at 1600 × g, to produce the plasma which was pipetted into cryovials and stored at −80 °C, before centrifugation and subsequent ultracentrifugation, as described above. For the septic patients plasma was obtained on the first day of admission to the Intensive Care Unit.

Exudate microparticles were prepared from skin blisters, generated by application of 0.1% cantharidin as previously described (27), and were harvested at the 24 h time point, because it corresponds to intense neutrophilic response (28, 29). Table II reports the demographics of these volunteers.

Proteomic Analysis—Microparticle extracts from two different preparations of each group were reduced in Laemmli sample buffer as described (12) and run in duplicate (four analyses for each set of microparticles). After separation by 5–20% tris-glycine polyacrylamide gel electrophoresis and silver-staining (PlusOne Silver staining kit, GE Healthcare), gel bands were subjected to in-gel digestion with trypsin using an Investigator ProGest (DIGILab) robotic digestion system. Tryptic peptides from the digests were separated on a nanoflow LC system (UltiMate3000, Thermo Fisher Scientific) and eluted with a 40 min gradient (10–25% B in 35 min, —40% B in 5 min, 90%
B in 10 min and 2% B in 30 min where A = 2% acetonitrile, 0.1% formic acid in high performance liquid chromatography (HPLC) H2O and B = 90% acetonitrile, 0.1% formic acid in HPLC H2O. The column (PepMap100 C18, 25-cm length, 75-µm internal diameter, 3-µm particle size, Thermo Fisher Scientific) was coupled to a nanospray source (Picoview) using RePlay (Advion) (30). Spectra were collected from a high-mass accuracy analyzer (LTQ Orbitrap XL, Thermo Fisher Scientific) using full MS scan mode over the mass-to-charge (m/z) range 450–1600. MS/MS was performed on the top six ions in each MS scan using the data-dependent acquisition mode with dynamic exclusion enabled. MS/MS peaklists were generated by extract_msn.exe and matched to human database (UniprotKB/Swiss-Prot Release 14.6, 20333 protein entries) using SEQUEST v.28 (rev. 13), (Bioworks Browser 3.3.1 SP1, Thermo Fisher Scientific) and X! Tandem, (Version 2007.01.01.2). Carboxyamidomethylation of cysteine was chosen as fixed modification and oxidation of methionine as variable modification. The mass tolerance was set at 50ppm for the precursor ions and at 1.0 AMU for fragment ions. Two missed cleavages were allowed. Scaffold (version 2.0.5, Proteome Software Inc., Portland, OR) was used to calculate the spectral counts and to validate MS/MS based peptide and protein identifications. According to the default values in the Scaffold software, the following peptide thresholds were applied; X! Tandem: -Log(Expect Scores) > 2.0, SEQUEST: deltaCn > 0.10 and Xcorr > 2.5 (2+), 3.5 (3+) and 3.5 (4+). Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (31). Protein identifications were accepted if they could be established at greater than 99.0% probability (32) with at least two independent peptides and a mass accuracy of ±10ppm of the precursor ion. Normalized spectral count for each proteins were obtained from at least two independent peptides and a mass accuracy of ±10ppm of the precursor ion. Normalized spectral count for each proteins were obtained from at least two independent peptides and a mass accuracy of ±10ppm of the precursor ion.

**Western Blot Analysis**—Presence of a select group of proteins identified by proteomic analysis was confirmed through standard SDS-PAGE, loading extracts from ~2 × 10^6 microparticles per lane (Millipore, Watford, UK). Western blot was conducted with specific antibodies against AnxA1 (5 µg/ml; clone 1B), anti-Alpha-2-macroglobulin (A2MG; 5 µg/ml clone 3D1; Thermo Scientific, Hampshire, UK), anti-Ceruloplasmin (CERU; 5 µg/ml; clone 8; BD Biosciences, Oxford, UK), anti-Heat shock protein 70 (hsp70; 5 µg/ml; clone 4E7, AB Serotec, Oxford, UK), anti-Lactoferrin (5 mg/ml; clone 1B), anti-A2MG (5 mg/ml; clone L3262, Sigma-Aldrich, Poole, UK) or anti-β-actin (ACTB; 5 µg/ml; clone AC-74, Sigma-Aldrich) overnight at 4 °C followed by a 1 h incubation with either an HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Dako, Cambridge, UK). Proteins were detected using an ECL detection reagent and visualized on Hyperfilm™ (GE Healthcare, Buckinghamshire, UK).

**Flow-cytometric Analysis**—To assess the homogeneity of the microparticle preparations, microparticles were suspended in PBS containing calcium and magnesium and incubated with either Alexa488 conjugated antibodies (following manufacturer’s instructions), mouse anti-human CD66b (clone: G10F5; BioLegend), CD14 (clone: M5E2, BD Biosciences), CD62P (clone: AK-4, BD Biosciences), CD41 (clone: HIP8, eBiosciences) or CD54 (clone: HCD54; Biolegend) fluorescently conjugated antibodies or relevant isotype controls for 20 min at room temperature and staining assessed using FACSCalibur or FACSCanTo II flow cytometers and data analyzed using either CellQuestTM software (Becton Dickinson) or FlowJo (Treestar Inc).

To determine microparticle cell surface protein expression, a double-staining protocol was applied using an anti-CD66b PE conjugated antibody (1:25) and one of the following Alexa488 conjugated antibodies: anti-AnxA1 (1 µg/ml; Clone 1B), anti-A2MG (5 µg/ml; Clone 3D1; Thermo Scientific), anti-CERU (2 µg/ml; Clone 8; BD Bioscience), or anti-HSP71 (2 µg/ml; Clone 4E7; Ab Serotec). All these antibodies and relevant isotype controls were labeled in house using monoclonal antibody conjugation kits (Invitrogen, Paisley, UK; cat no: A20181) following manufacturer’s instructions. In all cases, microparticles were incubated with the antibodies or relevant isotype controls for 45 min at 4 °C and before analysis with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuestTM software (Becton Dickinson). Protein abundance was assessed by subtracting the mean fluorescent value for each of the proteins of interest from the value obtained for the relevant isotype control.

In separate experiments, protein levels on neutrophil microparticles in plasma obtained from healthy volunteers and septic patients, along with exudates obtained from cantharidin-elicited skin blisters, were tested using staining protocols outlined above.

**Microparticle Functional Assays**

**Reactive Oxygen Species Determination—Lucigenin assay.** Microparticles (~6 × 10^6) obtained from both FIP and ImP populations were pre-incubated in the presence of 5 µM Lucigenin for 10 min at 37 °C prior to incubation with PMA (16 µM; Merck Chemicals Ltd., Nottingham, UK) or vehicle, and luminescence was assessed in the thermostated chamber of a luminometer (Wallac VICTORZ 1420 Multilabel Counter, Perkin Elmer Life Science) for further 45 min at 37 °C. DCFDA assay. FIP and ImP microparticles were suspended at 2 × 10^6 per ml in HBSS and incubated with or without pertussis toxin (1 µg/ml) for 3h at room temperature. These were then incubated with 10 µM DCFDA (30 min at 37 °C). A basal fluorescence reading in the FL1 channel of a FACSCalibur flow cytometer (Becton Dickinson) using CellQuestTM software (Becton Dickinson) was taken prior to stimulation with fMLF (1 µM) or vehicle. Leukotriene (LT)B4 Generation—FIP or ImP microparticles (~6 × 10^6) were resuspended in Krebs Ringer bicarbonate buffered solution, prior to stimulation with 100 nM arachidonic acid (Calbiochem) or with or without 10 nM A23187 (Sigma-Aldrich). After a further 15 min at 37 °C, LTB4 levels were measured using a Parameter™ LTB4 assay kit (R&D Systems, Abingdon, United Kingdom).

**Chemotaxis—ImP or FIP microparticles were suspended at ~3 × 10^7 per ml in RPMI1640 that was filtered through a 0.2 µm filter (without FCS or supplements) and the extent of chemotaxis toward fMLF (1 µM) was assessed. Therefore, 27 µl of a solution of the chemotactic agents or a vehicle control were added to the bottom of a 96-well ChemoTx® plate equipped with 2 µm pore filters (Neuro-probe, Gaithersburg, USA) and the microparticle containing solution was added to the top. After 1h at 37 °C the remaining solution at the top of the chemotaxis chamber was removed and the membrane washed once with RPMI1640. Subsequently, the number of microparticles in the lower chamber was quantified using a previously calibrated flow cytometer as a function of the AnxAV positive events within the microparticle gate.

**Microparticle Sensing by Endothelial Cells**

**Microarray Studies**—HUVECs were grown to monolayers in six-well plates and co-incubated with buffer, FIP or ImP microparticles (~8 × 10^6) for 6h (at 37 °C and 5% CO2). RNA was subsequently extracted using an RNaseasy® Plus mini kit (Qiagen, West Sussex, UK) following manufacturer’s instructions. Three independently prepared HUVEC and microparticle preparations were employed for this analysis. RNA integrity was determined on RNA 6000 Nano LabChips (Agilent Technologies, Palo Alto, CA). Preparation of cRNA and hybridization on the whole genome microarrays HumanHT-12 v4 Expression BeadChip were performed according to the manufacturer’s protocols using Custom Illumina® TotalPrepTM Kit and Whole-Genome Gene Expression Direct Hybridization Assay (Illumina, Essex, UK). Raw measurements were processed by GenomeStudio software (Illumina). GenomeStudio checks that a probe has ≥3 beads present on the array (if not, the probe is considered to be missing), does a local background subtraction and condenses bead-level data into a single
Heterogeneity in Neutrophil Microparticles

probe-level value per probe by removing outliers >3 median absolute deviations from the median, recalculating the mean of the remaining values. Data were quantile normalized and fold change expressed as the ratio treatment/control. Genes were considered to be significantly modulated if the mean signal obtained in either of the microparticle-treated groups was significantly higher ($p < 0.05$) than that obtained for the DPBS treated group. Unsupervised hierarchical clustering (heat map) was performed using Babelomics 4 (33). All microarray data are publicly available in the Gene Expression Omnibus (GEO) database with accession number GSE25154.

Quantitative Real-time PCR (qRT-PCR)—Results of the microarray data were validated by qRT-PCR. Real time-PCR was performed with 120 ng of cDNA per well, 1 μl primers and Power SYBR Green PCR Master Mix (Applied Biosystems, UK), using the ABI Prism 7900HT Sequence Detection System. Predesigned QuantiTect® Primer Asays (Qiagen) were used to measure the expression levels of: ATF3, CASP2, COX5A, COX5B, CXCL1, CXCL2, CXCL8, GAPDH, ICAM1, ITGB4, ICAM5, NLRP3, NLRP6, NOX4, NRG1, NRG1, PECAM1, PPT1, PTPRG, THBS1, and VCAM1, and absence of unspecific products was assessed by including a dissociation step. Gene expression values were calculated as log$_2$(2$^{-\Delta\Delta C_t}$) using GAPDH as endogenous control.

Functional Analysis—Functional analyses of identified proteins and the differentially expressed genes were performed using PHANTHER classification system v6.1 ((34)). The top 15 (for the proteomics analysis) or top 10 (gene analysis) pathways according to significance were selected.

In Silico Prediction of Drugs Connections—The two distinct gene expression signatures generated in this study (HUVEC cells treated either with ImP or FIP microparticles) were compared with the more than 7000 gene expression profiles obtained from 1309 small molecules contained in the current version (build 02) of the Connectivity Map (CMap) (35)). The similarity between the gene expression profiles of interest and those contained in the CMap database is determined by the connectivity score, ranging from $-1$ to $+1$. Considering the hypothesis that if a drug has a gene expression signature that is similar to another drug could potentially be used to identify novel mechanisms of action, we focused on the analysis of the drugs showing a positive score. The top 10 drugs with the highest score were selected.

Statistical Analyses—Experiments were performed in triplicate and data are expressed as Mean ± S.E. Statistical differences were determined using one-way analysis of variance or Student’s t test as appropriate, using GraphPad Prism™. A probability value less than 0.05 was taken as significant for rejection of the null hypothesis.

RESULTS

Neutrophil Microparticle Proteomic Characterization

ImP and FIP Microparticle Microparticles—Neutrophil microparticles can induce distinct effects on recipient cells by promoting either activating/pro-inflammatory (24, 25) or inhibiting/anti-inflammatory effects (12, 24–26, 36). Here we investigated whether these multiple actions of neutrophil microparticles resulted from a distinct proteomic profile. Freshly prepared neutrophils were incubated either in suspension (FIP) or over a HUVEC monolayer (ImP) for 20 min prior to stimulation. Flow cytometry was employed to confirm the presence of microparticle in our preparations, staining against the CD66b antigen (11), and calibrating against 1-μm beads (Fig. 1A). Over 95% of the counts were CD66b$^+$ in both preparations (Fig. 1B) with no detectable contribution of platelet, monocytes or endothelial microparticles in these preparations (supplemental Fig. S1) as determined by flow-cytometric staining for CD62P, CD41 (platelet), CD14 (monocyte), and CD54 (endothelial) positive microparticles (supplemental Fig. S1). Adherent neutrophils were found to produce a larger number of microparticles (ImP microparticles) than neutrophils stimulated in suspension (FIP microparticles). Of note there were no significant differences in overall size as determined by the forward and side scatter parameters in flow cytometry between these two subsets (Fig. 1A). These results suggest that the microparticle populations obtained under these conditions displayed similar physical characteristics with no detectable contribution by other cell types.

Proteomics Analysis of ImP and FIP Microparticles—Tandem gel-LC-MS-MS proteomic analysis was performed on two distinct microparticle preparations from two separate donors. Each of the microparticle preparations was then assayed in duplicate to account for any intrarun variation in the analysis. In this proteomic analysis we identified 342 proteins in the ImP microparticles and 304 proteins in the FIP microparticles. Protein expression, as determined by spectral counting, in each of the microparticle subsets was found to be similar between the two microparticle preparations suggesting minor intra donor variation (Table I). In addition spectral counts were also found to be similar in each of the duplicate runs performed on the microparticle preparations. Around 30% of the proteins were uniquely expressed in one of the microparticle subsets (Fig. 1B), suggesting that culture conditions might influence the way microparticles are generated from the same cell type. Table I lists the most abundant 20 proteins in each microparticle subset and a full list can be found in supplemental Table S1. The identified peptides are detailed in supplemental Table S2. Among the most abundant proteins contained in both microparticles populations (223), molecules such as myeloperoxidase, ANXA1, cathepsin G, or S100A8 (Fig. 1B) are typical for this leukocyte type.

A functional analysis of canonical pathways was performed on each microparticle subset (Fig. 1C). The pathways significantly associated with both subsets were mainly immune-related pathways: integrin signaling, inflammation mediated by cytokines and chemokines or EGF and FGF signaling pathways. Despite the important difference in their protein profile, this type of functional analysis did not reveal any evident difference between both microparticle subsets, possibly because it only accounts for the number of proteins involved in a given pathway regardless of expression level and specific role in the pathway—activator/repressor—and hence it has limited utility. However in both cases the functional analysis suggests that both microparticles subsets might have immune-modulatory functions.

Determining the Expression Profiles of a Select Group of Identified Proteins in the Two Microparticle Subsets—We next assessed the abundance of a select group of proteins identified by the proteomic screen in the two microparticle subsets by Western blotting. To better evaluate protein expres-
Heterogeneity in Neutrophil Microparticles

Fig. 1. Differential stimulation of neutrophils yields microparticles with a distinct proteome. A, The physical properties of microparticles obtained from neutrophils after stimulation in fluid-phase (FIP; in suspension) or immobilized-phase (ImP; post adhesion to a HUVEC monolayer) were assessed employing the forward and side scatter parameters on the dot-plot generated by flow-cytometric analysis. The origin from the neutrophil was ascertained by staining the microparticles by anti-CD66b staining. B, Venn diagrams representing the proteomic content identified in each of the neutrophil microparticle subsets as determined using tandem LC-MS-MS. C, Ingenuity Pathway Analysis software was used to highlight the top 15 functions of the various proteins expressed in the distinct microparticle subsets as illustrated. In all cases results are representative of four distinct analyses.

sion in each of the two-microparticle subsets we loaded equal number of microparticles for each of the subsets. Here we found that that alpha-2-macroglobulin (A2MG) and ceruloplasmin (CERU) were enriched in the ImP microparticles, while heat shock 70kDa protein 1 (HSP71) was elevated in FlP microparticles (Fig. 2A). Annexin A1 (ANXA1), Lactoferrin (TRLF), and β-actin (ACTB) were expressed at equal levels in the two microparticles subsets. Densitometric analysis employing ACTB as a loading control further corroborated the relative protein distribution in these two microparticle subsets (Fig. 2A). Flow cytometric assessment of this select group of proteins demonstrated that A2MG and CERU were present on
Heterogeneity in Neutrophil Microparticles

| Sample          | Protein name                  | UNIProt ID | Molecular weight (kDa) | p value (t-test ImP vs FIP) | AVG Spectral counts | S.E. Spectral counts |
|-----------------|-------------------------------|------------|------------------------|--------------------------|---------------------|----------------------|
| **ImP Microparticles** |                               |            |                        |                          |                     |                      |
| Lactotransferrin | TRFL                          | 78         | 0.027                  | 829.3                    | 83.2                |
| Alpha-2-macroglobulin | A2MG                      | 163        | 0.0000014              | 723.3                    | 49.1                |
| Myeloperoxidase  | PERM                         | 84         | 0.081                  | 440.3                    | 35.6                |
| Ig mu chain C region | IGHM                     | 49         | 0.00019                | 317.3                    | 33.7                |
| Serum albumin    | ALBU                         | 69         | 0.0037                 | 310.0                    | 15.7                |
| Haptoglobin      | HPT                          | 45         | 0.0002                 | 203.8                    | 20.8                |
| Integrin alpha-M | ITAM                        | 127        | 0.27                   | 202.5                    | 12.2                |
| Serotransferrin  | TRFE                         | 77         | 0.00016                | 161.3                    | 19.7                |
| Annexin A6       | ANXA6                        | 76         | 0.48                   | 131.5                    | 8.2                 |
| Apolipoprotein B-100 | APOB                   | 516        | 0.0000044              | 127.8                    | 12.8                |
| Hemoglobin subunit beta | HBB                   | 16         | 0.74                   | 112.8                    | 13.8                |
| Ig gamma-1 chain C region | IGHG1           | 36         | 0.0000072              | 108.8                    | 7.5                 |
| Histone H4       | H4                           | 11         | 0.049                  | 107.8                    | 23.3                |
| Hemoglobin subunit alpha | HBA                | 15         | 0.55                   | 107.0                    | 5.6                 |
| Complement C3    | CO3                          | 187        | 0.00021                | 97.0                     | 15.3                |
| Actin, cytoplasmic 1 | ACTB               | 42         | 0.019                  | 95.0                     | 7.2                 |
| Annexin A1       | ANXA1                        | 39         | 0.19                   | 93.3                     | 3.0                 |
| Integrin beta-2  | ITB2                         | 85         | 0.65                   | 91.3                     | 6.6                 |
| Myosin-9         | MYH9                         | 227        | 0.49                   | 87.0                     | 16.7                |
| Ceruloplasmin    | CERU                         | 122        | 0.0004                 | 85.3                     | 14.6                |
| **FIP Microparticles** |                               |            |                        |                          |                     |                      |
| Lactotransferrin | TRFL                         | 78         | 0.027                  | 852.3                    | 26.3                |
| Myeloperoxidase  | PERM                         | 84         | 0.081                  | 469.3                    | 44.2                |
| Integrin alpha-M | ITAM                        | 127        | 0.27                   | 172.8                    | 16.2                |
| Serum albumin    | ALBU                         | 69         | 0.0037                 | 149.8                    | 5.3                 |
| Histone H4       | H4                           | 11         | 0.049                  | 123.3                    | 6.4                 |
| Annexin A6       | ANXA6                        | 76         | 0.48                   | 107.3                    | 13.9                |
| Actin, cytoplasmic 1 | ACTB               | 42         | 0.019                  | 104.8                    | 15.1                |
| Myosin-9         | MYH9                         | 227        | 0.49                   | 103.3                    | 47.7                |
| Annexin A1       | ANXA1                        | 39         | 0.19                   | 101.3                    | 19.4                |
| Catalase         | CATA                         | 60         | 0.0023                 | 98.8                     | 12.9                |
| Protein S100-A8  | S10A8                        | 11         | 0.0021                 | 88.5                     | 3.8                 |
| Plastin-2        | PLSL                         | 70         | 0.034                  | 88.5                     | 29.5                |
| Hemoglobin subunit beta | HBB           | 16         | 0.74                   | 88.0                     | 7.1                 |
| Matrix metalloproteinase-9 | MMP9       | 78         | 0.056                  | 78.3                     | 5.3                 |
| Annexin A3       | ANXA3                        | 36         | 0.002                  | 76.3                     | 4.5                 |
| Hemoglobin subunit alpha | HBA       | 15         | 0.55                   | 75.0                     | 5.1                 |
| Annexin A11      | ANX11                         | 54         | 0.38                   | 73.3                     | 15.0                |
| Integrin beta-2  | ITB2                         | 85         | 0.65                   | 69.8                     | 9.0                 |
| Heat shock 70 kDa protein 1 | HSP71     | 70         | 0.031                  | 68.5                     | 15.3                |
| Glyceraldehyde-3-phosphate dehydrogenase | G3P | 36 | 0.0046 | 62.0 | 5.6 |

the surface of ImP microparticles, HSP71 levels were more abundant on FIP microparticles whereas ANXA1 was equally expressed on the surface on both microparticle subtypes (Fig. 2B). These results corroborate the finding that distinct neutrophil stimulation yields microparticles with a characteristic proteomic profile.

Are ImP and FIP Microparticles Produced in Human Inflammation?—To determine the translational value of our observations to the in vivo clinical scenario, we measured expression of a select number of proteins under two distinct inflammatory conditions. The exudates from experimental cantharidin-elicited skin blister model, which leads to formation of a highly neutrophilic response and plasma from patients suffering from severe sepsis or septic shock resulting from community acquired pneumonia (CAP), these were compared with plasma neutrophil microparticles obtained from healthy volunteers (Table II).

Flow-cytometric analysis for expression of A2MG, CERU, HSP71 and ANXA1 on the surface of neutrophil microparticles demonstrated that CD66b+ microparticles harvested from blister exudates contained higher levels of HSP71 (13.8 ± 0.5% versus 5.7 ± 0.2%), CERU (22.2 ± 0.7% versus 5.6 ±
0.1%) and ANXA1 (12.8 ± 0.4% versus 5.7 ± 0.2%) when compared with healthy volunteer plasma neutrophil microparticles (Fig. 3). On the other hand, elevated levels of A2MG (14.6 ± 0.3% versus 5.2 ± 0.1%) and CERU (13.4 ± 0.5% versus 5.6 ± 0.1%) were observed in plasma neutrophil microparticles from CAP patients, with a significant reduction in HSP71 and ANXA1 expression in relation to blister-derived and healthy volunteer plasma microparticles (Fig. 3). These results confirm that selected proteins identified in our proteomic profiling are regulated in vivo and can indeed be expressed by human neutrophil microparticles under both homeostatic conditions and during local or systemic inflammatory responses.

**ImP and FIP are Also Effectors of Inflammation**—We next investigated whether these microstructures were mere vehicles carrying proteins from source to sink, or whether they could act as effectors in their own respect. Analysis of the proteome highlighted the presence of various components of the NADPH oxidase complex in the FIP microparticles including NCF2 (p67phox) and NCF4 (p40phox; supplemental Table S1 and Table II). We also found evidence for the presence of CY24B (NOX2; supplemental Tables S1 and S2) and NCF1

---

**Table II**

| Sample               | PMN counts (ml⁻¹) | Exudate volume (ml) | Age (years) | Sex   | CD66b⁺ microparticles (%) | Total microparticle count (ml⁻¹) |
|----------------------|-------------------|---------------------|-------------|-------|--------------------------|--------------------------------|
| Healthy volunteer   | 6.70 × 10⁶ ± 1.60 × 10⁶ | Not applicable       | 30.0 ± 0.5  | 9 M/7F| 6.5 ± 4.5                | 29567 ± 2071                   |
| Blister exudate     | 0.62 × 10⁶ ± 0.22 × 10⁶ | 1.15 ± 0.65         | 31.3 ± 0.4  | 6 M/4F| 39.9 ± 11.5              | 63367 ± 1423                   |
| CAP sepsis plasma   | 10.27 × 10⁶ ± 0.51 × 10⁶ | Not applicable       | 58.8 ± 1.6  | 2 M/8F| 12.3 ± 5.2               | 40093 ± 1670                   |

---

**Fig. 2.** Stimulus-dependent protein expression in neutrophil microparticles. (A), Western blotting selecting proteins that were either predominantly (alpha-2-macroglobulin, A2MG; ceruloplasmin, CERU) or uniquely (heat shock 70kDa protein 1, HSP71) expressed in one of the microparticle subsets, or expressed in both sets (Annexin A1, ANXA1; Lactoferrin, TRLF and β-actin, ACTB). ACTB was used as a loading control for densitometry analysis. and (B) flow cytometric analyses of a select group of proteins identified in the proteomic screen (see Methods for details). Results are mean ± S.E. of n = 3–4 distinct microparticle preparations.
These findings prompted us to investigate whether FIP micro-
particles produced reactive oxygen species (ROS) upon direct
stimulation. Addition of 1 μM fMLF to FIP microparticles led to
ROS production, monitored as increase in fluorescence, an
action that was not shared by ImP microparticles, (Fig. 4A).
Addition of pertussis toxin blocked ROS production in re-
sponse to fMLF (Fig. 4B) indicating that this was a receptor-
mediated response.

The proteomic screen also identified leukotriene A4 hydro-
lase (LKHA4) in the proteome of FIP microparticles (supple-
mental Tables S1 and S2) and indicated the presence of
5-lipoxygenase (5-LOX) in these microparticles (supplemental
Fig. S1B). Thus we investigated whether the microparticle
subsets identified herein could generate LTB₄ on stimulation.
Of note, both microparticle subsets produced LTB₄ in the
presence of arachidonic acid (732 ± 82 pg/ml and 820 ± 58
pg/ml for FIP and ImP microparticles respectively), a process
that was enhanced when microparticles were incubated in
presence of the calcium ionophore A23187 (Fig. 4C).

Because the proteomic screen demonstrated presence of
37 cytoskeletal related proteins in ImP microparticles and 40
in FIP microparticles including Tubulin Beta-5 Chain, β-Actin
(ACTB), and Myosin 6 (supplemental Tables S1 and S2), we
next investigated whether these microparticles could migrate
down a chemotactic gradient. Incubation of microparticles in
the presence of buffer alone (Fig. 4D). Together these
results suggest that microparticles may move to specific tis-
sue sites and exert independent effector functions.

Neutrophil Microparticles Elicit Distinct Gene Expression
Profiles in Endothelial Cells—Several mechanisms have been
reported to explain how microparticles exert their biological
actions and how they influence cellular processes (see Intro-
duction). They can interact with surface molecules on a target
cell, they can transfer their contents to a target cell by fusion
or phagocytosis or they can directly produce ROS, as we
showed earlier. Here we next investigated a novel mechanism
that is whether microparticles actively modulated the gene
expression pattern of target cells producing longer-lasting
effects rather than just a passive transfer of their contents. On
these bases, we set out to explore whether ImP and FIP
microparticles subsets could also modify the gene expression
profile of endothelial cells.

As shown in Fig. 5A, microparticles had a significant impact
on the gene expression pattern of HUVEC cells: 501 and 1154
genes were significantly modulated in endothelial cells when
co-incubated with ImP and FIP microparticles, respectively
(see also Table III and supplemental Table S3). A total of 251
genes were significantly altered in both conditions whereas a
substantial number of genes were affected only by one of the
microparticle subsets. The triplicates used in our study
showed very high consistency (Fig. 5B) and a real time-PCR
confirmation of selected genes showed an 87% correlation
between microarray and PCR data, validating the microarrays
analysis. In line with the results obtained for the microparticle
proteomes, a functional analysis of canonical pathways did
not reveal major differences between both microparticles

**Fig. 3.** Microparticle heterogeneity in human samples. Expression levels of A2MG, CERU, HSP71, ANXA1 in CD66b+ microparticles in plasma obtained from healthy volunteers (HV) or septic patients (Sep), as well as in exudates obtained from cantharidin-elicited skin blisters (BL). Data are mean ± S.E. of 10–16 samples tested in duplicate. *p < 0.05, ** p < 0.01 versus CT group; †† p < 0.01 versus respective BL group.
treatments, however it indicated that the genes affected are mainly related to immune responses, such as integrins and cytokines as well as angiogenesis and apoptosis.

A detailed study of the genes altered in each subset uncovered important differences: the up-regulation of pro-inflammatory genes by ImP microparticles, such as IL1β, CCL3L1, or STAT3, together with the down-regulation in FlP of genes such as STAT1, NFKB1Z, CCL8, or CXCL6 suggest a pro-inflammatory phenotype for ImP microparticles and an anti-inflammatory phenotype for FlP microparticles. In addition, incubation of ImP microparticles with HUVEC led to down-regulation of genes with important roles in vascular function including adrenomedullin (ADM), a protective factor for blood vessels, apelin (APNL) which participates in the control of blood pressure, or the enzyme serum and glucocorticoid regulated kinase 1 (SGK1), which regulates endothelial cells apoptosis. On the contrary, protective factors were up-regulated by the FIP subset, including ANGPTL4 that acts as an endothelial cell survival factor, and CD55 (an anti-inflammatory receptor for complement).

Finally, we performed an in silico comparison of our gene expression profiles, with those produced by more than 1300 drugs contained in The Connectivity Map (CMap) database. We found that the top 10 drugs with the highest score, that is, with a similar gene expression profile in the FIP microparticles (Table IV), included an immunosuppressant (phenanthriodione), a cytoprotective agent (16,16-dimethylprostaglandin 1(Prostaglandin 1(Prostaglandin 1(Prostaglandin 1(Prostaglandin E2)), and the anti-inflammatory drugs SC-560 and NS-398 (selective COX-1 and COX-2 inhibitors, respectively), providing further evidence of the potential anti-inflammatory nature of the FIP microparticles. On the other hand, although not on the top 10 drugs shown in Table IV, several antimicrobial/antifungal drugs were positively associated with ImP microparticles-treated HUVEC, such as ikarugamycin (score 0.371), josamycin (0.366), thiamphenicol (0.353), furazolidone (0.302), vancomycin (0.281), or amphotericin B (0.279).

**DISCUSSION**

We report herein that neutrophil stimulation can lead to the generation of heterogeneous microparticles characterized by distinct proteomes. This difference in protein composition...
confers characteristic functional abilities to microparticles (e.g., the ability of FIP microparticles to produce ROS). In addition, microparticles produced by the same cell type in response to different stimuli (i.e., in adhesion versus in suspension) induced discrete gene expression profile once added to recipient cells.

Since their discovery there has been considerable progress in appreciating that cell-derived microparticles may play an active role in homeostasis and disease (3, 37–39). Recent evidence suggests that microparticles exert potent actions in cell-to-cell communication in both normal physiology and disease. To date, most studies have focused on the cellular origin of microparticles during disease (for a comprehensive review on endothelial microparticles see (14)) whereas only limited information is available on the protein composition of these microstructures, as well as their potential downstream effects.

**Fig. 5.** Neutrophil microparticles modify endothelial cell gene expression profile. HUVEC were incubated with vehicle, FIP or ImP microparticles (~8 × 10⁶) for 6 h at 37 °C. After cell harvest and RNA extraction, gene expression profile was assessed using Illumina HT12v4 microarrays. A, Venn diagram showing the number of significantly (p < 0.05) regulated genes in the ImP and FIP microparticles treated versus vehicle cells (PBS). Selected genes are shown. B, Heatmap generated with the individual replicates showing intersample similarity. C, Real-time-PCR validation of 15 distinct genes identified in the microarray analysis, showing very high correlation between the two techniques. Results are cumulative from three separate experiments with distinct microparticle and HUVEC preparations. **(D, E)** Functional analysis showing the top 15 pathways associated with ImP and FIP microparticles treated HUVEC respectively.
actions once released into the surrounding milieu by the parent cells. In line with previous studies conducted with a human monocytic cell line and endothelial cells (9, 40) we provide evidence for heterogeneity in neutrophil microparticles produced in response to discrete stimuli. In line with our findings Tímár and colleagues recently demonstrated that only in response to opsonized *S. aureus* neutrophils produced microparticles endowed with antibacterial properties (36).

One of the initial steps in the activation cascade of a bloodborne neutrophil that leads to its recruitment to the site of inflammation is interaction with venular endothelium to begin the process of extravasation (41, 42). Indeed, indication for microparticle generation during neutrophil migration across the endothelial wall has recently been provided (23), further underscoring the importance of establishing the content of these microparticles. Furthermore, evidence from clinical studies in a number of diseases suggests that neutrophil microparticles may be useful biomarkers in a number of inflammatory pathologies. For example, in conditions such as vasculitis and severe injury, plasma CD66b+ microparticle

| Sample | Gene name | Symbol | Fold change | p value |
|--------|-----------|--------|-------------|---------|
| ILMN_1758895 | Cathepsin K | CTSK | 40.5 | 0.0001 |
| ILMN_2079098 | INTS3 and NABP interacting protein | C9orf80 | 46.8 | 0.0000 |
| ILMN_1773337 | Dickkopf 1 homolog (Xenopus laevis) | DKK1 | 46.8 | 0.0000 |
| ILMN_1844593 | — | — | 52.4 | 0.0000 |
| ILMN_1739393 | Selectin E | SELE | 53.8 | 0.0000 |
| ILMN_1699651 | Interleukin 6 | IL6 | 54.3 | 0.0000 |
| ILMN_1793025 | Mitochondria-localized glutamic acid-rich protein | OSAP | 64.3 | 0.0000 |
| ILMN_1794782 | ATP-binding cassette, sub-family G, member 1 | ABCG1 | 70.6 | 0.0000 |
| ILMN_2355033 | KIAA1147 | KIAA1147 | 45.6 | 0.0000 |
| ILMN_1658094 | Zinc finger protein 365 | ZNF365 | 42.5 | 0.0001 |
| ILMN_1768534 | Basic helix-loop-helix family, member e40 | BHLHB2 | 39.1 | 0.0001 |
| ILMN_1775501 | Interleukin 1, beta | IL1B | 39.0 | 0.0001 |
| ILMN_2189027 | Lipase, endothelial | LIPG | 38.9 | 0.0001 |
| ILMN_1689037 | Lipase, endothelial | LIPG | 38.1 | 0.0002 |
| ILMN_3253456 | Fibronectin type III domain containing 3B | FNDC3B | 38.1 | 0.0002 |
| ILMN_1801584 | Chemokine (C-X-C motif) receptor 4 | CXCR4 | 36.8 | 0.0002 |
| ILMN_1699695 | Tumor necrosis factor receptor superfamily, member 21 | TNFRSF21 | 36.1 | 0.0002 |
| ILMN_1747759 | WD repeat and SOCS box containing 1 | WSB1 | 36.7 | 0.0002 |
| ILMN_2117323 | Phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 beta | PIK3C2B | 40.0 | 0.0001 |
| ILMN_1758895 | Cathepsin K | CTSK | 40.5 | 0.0001 |
| ILMN_2079098 | INTS3 and NABP interacting protein | C9orf80 | 46.8 | 0.0000 |
| ILMN_1773337 | Dickkopf 1 homolog (Xenopus laevis) | DKK1 | 46.8 | 0.0000 |
| ILMN_1844593 | — | — | 52.4 | 0.0000 |
| ILMN_1739393 | Selectin E | SELE | 53.8 | 0.0000 |
| ILMN_1699651 | Interleukin 6 | IL6 | 54.3 | 0.0000 |
| ILMN_1793025 | Mitochondria-localized glutamic acid-rich protein | OSAP | 64.3 | 0.0000 |
counts are elevated (43, 44). However, it is difficult to understand the potential influence that such microparticles could be exerting without first appreciating their composition.

For all these reasons, herein we focused on the neutrophil microparticle proteome employing two distinct culture conditions with freshly prepared neutrophils. This yielded microparticles with similar physical properties, as deduced by flow cytometry. Proteomic analysis of these microparticle subsets demonstrated that they possessed distinct proteomic profiles with about 30% of the total proteins identified for the two subsets being uniquely expressed in one of the microparticle subsets. Protein expression profiles obtained by Western blotting for a select group of proteins identified during the proteomic screen corroborated the results obtained by LC-MS-MS, both with respect to their identity and distribution between the two microparticle groups. The detection of these proteins was highly reproducible in view of the four samples analyzed by proteomics (see Methods) but also the several assays of Western blotting performed: some of the blots are reported in here, but have been reproduced (e.g. for ANXA1) in over 12 distinct preparations of neutrophil microparticles (data not shown). In addition we propose that both ANXA1 and TRLF may be employed as sensitive loading controls in these microparticle subsets because they were equally expressed in both microparticle subsets and found to be at higher levels than β-actin. These findings extend those made in our initial study that focused on ANXA1+ microparticles (detected in adherent—but not resting—conditions; see ref (12.), that we found to mediate, at least in part, the acute nongenomic anti-inflammatory properties exerted by this neutrophil microparticle subset (9). The observation that neutrophils have the ability to respond to a specific stimulus by producing microparticles loaded with a distinct proteomic profile supports the notion that microparticle production is a regulated process and that they might be endowed with very discrete functions, as shown herein and discussed below. The hypothesis is further supported by the finding that neutrophil microparticles also exert antibacterial properties (36). It is noteworthy that proteins known to be expressed on neutrophil microparticles such as L-selectin (CD62L) and CD66b were not identified in our proteomic screen. This may be due to a low expression of these proteins in the two microparticle subsets investigated herein when compared with the identified proteins. In addition these proteins are also known to undergo glycosylation, a modification that not only influences their ability to migrate in the SDS-gel but also decreases the probability for mass spectral identification.

To better appreciate the translational potential of our findings we next investigated whether the proteins identified herein were also expressed on neutrophil microparticles obtained from three distinct human settings. Here we assessed microparticles from skin blister exudates since this characterized by a highly neutrophilic inflammation (28), predicting it might reflect the FlP microparticle phenotype. We also profiled plasma microparticles from patients suffering from sepsis, tested as a sample likely to yield ImP-like microparticles, in view of the central role of endothelial activation in this disease (45). The data obtained satisfied to a large extent this

| Sample       | Drug                                       | Activity                                      | Score | n  | p value | % non-null |
|--------------|--------------------------------------------|-----------------------------------------------|-------|----|---------|------------|
| ImP Microparticles | 12,13-EODE (iso-leukotoxin) | Produced by neutrophils during oxidative burst | 0.697 | 1  | —       | 100        |
|              | 5162773                                  | Unknown                                      | 0.676 | 1  | —       | 100        |
|              | (-)-Catechin                             | Antioxidant                                  | 0.654 | 1  | —       | 100        |
|              | Cytochalasin B                           | Antimotic                                    | 0.555 | 1  | —       | 100        |
|              | Topiramate                               | Anti-covalasulant                            | 0.545 | 1  | —       | 100        |
|              | 5151227                                  | Unknown                                      | 0.535 | 1  | —       | 100        |
|              | Tomelukast                               | Leukotriene D4 antagonist                    | 0.521 | 1  | —       | 100        |
|              | 5186324                                  | Unknown                                      | 0.516 | 1  | —       | 100        |
|              | Pralidoxime                              | Cholinesterase reactivator                   | 0.477 | 4  | 0.03617 | 75         |
|              | 5213008                                  | Unknown                                      | 0.465 | 1  | —       | 100        |
| FlP Microparticles | Phenanthridinone                        | Immunosuppressant                            | 0.644 | 1  | —       | 100        |
|              | Topiramate                               | Anti-covalasulant                            | 0.636 | 1  | —       | 100        |
|              | Demecolcine                              | Anticancer                                   | 0.574 | 1  | —       | 100        |
|              | 2-deoxy-D-glucose                         | Glycolysis inhibitor                         | 0.550 | 1  | —       | 100        |
|              | 16,16-dimethylprostaglandin E2           | Cytoprotective                               | 0.523 | 3  | 0.00176 | 100        |
|              | Naltrexone                               | Opioid antagonist                            | 0.482 | 5  | 0.00192 | 80         |
|              | SC-560                                   | Selective COX-1 inhibitor                    | 0.473 | 3  | 0.08261 | 66         |
|              | NS-398                                   | Selective COX-2 inhibitor                    | 0.448 | 3  | 0.09723 | 66         |
|              | NS-295                                   | Unknown                                      | 0.438 | 2  | 0.50195 | 50         |
|              | Mevalolactone                            | Anti-oxidant                                 | 0.436 | 3  | 0.08749 | 66         |
Heterogeneity in Neutrophil Microparticles

Intriguingly, we also found that microparticles could also move in response to a chemotactic gradient (Fig. 4), in line with the identification of several cytoskeletal proteins in the microparticle proteome (supplemental Table S1). This suggests that microparticles may be able to specifically migrate to inflammatory loci possibly reaching relatively distant target cells to affect downstream inflammatory events. In addition, our results suggest that incubation of neutrophils with fMLF leads to the functional incorporation of formyl peptide receptors (FPR) to microparticles. Engagement of GPCRs by their cognate agonists may lead to receptor internalization where the receptor may either be targeted for degradation or recycled to the cell surface (49). Our finding suggest that FPR receptors may be recycled to the cell surface following agonist binding where in turn it may be incorporated into microparticles. Moreover, these data suggest that receptor signaling in microparticles follows distinct dynamics to those found in the parent cells which will need to be explored in more detailed future studies.

LTB4 is a lipid mediator biosynthesized via the conversion of arachidonic acid by the actions of two enzymes, 5-lipoxygenase and leukotriene A4 hydrolase. LTB4 is a potent neutrophil chemo-attractant leading to neutrophil chemotaxis, aggregation, and transmigration across the epithelium and/or endothelium to the site of inflammation. The ability of both microparticle subsets to produce LTB4 on stimulation is noteworthy in the context of an orchestrated inflammatory response, suggesting that microparticles can propagate the production of “danger signals.” Equally possible, is that the persistence of these microparticles within the vasculature or a given tissue (e.g. rheumatoid arthritis joint) could prove to be detrimental and lead to chronic disease (43, 50).

We concluded the present study by determining if the unique proteomic content of the two microparticle preparations could be sensed, at the genetic level, by recipient cells thus opening the possibility for long-lasting downstream actions of these vesicles. Both microparticle subsets regulated genes that are involved with mitosis, intracellular signaling protein transport and oxidative phosphorylation among others. Of note incubation of FIP with endothelial cells produced the most profound changes with respect to the number of genes differentially expressed and the resultant biological processes affected. These experiments demonstrate that microparticles with different proteomes, albeit generated from the same cell type for the same donor, can elicit profound alterations in gene expression. To better appreciate the functional relevance of this response we used a recently developed tool, the CMap database, that compares the gene expression profiles to those elicited by known drugs. This demonstrated that FIP microparticles induced a gene signature that is reminiscent of molecules with an inhibitory—if not anti-inflammatory, e.g. the positive association with COX inhibitors—profile (Table IV). In contrast, incubation of endothelial cells with ImP microparticles induced a gene signature that more resembled the gene print of antibiotics and other drugs able to fight infections. Furthermore assessment of the proteins identified in ImP microparticles supports the notion that these microparticles may exert antibacterial properties (36). These proteins include A2MG and the complement proteins C03 and C04A that were mainly or solely identified in ImP microparticles, and the proteins BPI (bacterial permeability-increasing protein), DEFA3 (neutrophil defensin 3) or OLFM4 (olfactomedin 4), which show higher expression in ImP microparticles.

Neutrophils can orchestrate a variety of responses that go well beyond their classical function of killing bacteria through ROS and proteolytic enzyme release. These cells can produce cytokines and mediators able to influence downstream responses evoked by macrophages, dendritic cells and lymphocytes (see ref (41) for a review). In addition neutrophil microparticles also regulate macrophage and dendritic cell
responses in the presence of pro-inflammatory stimuli via the Mer receptor tyrosine kinase (MerTK) and PI3K/Akt pathways (51). Neutrophil microparticles also stimulate the efferocytosis of apoptotic cells (1, 52) and the biosynthesis of pro-resolving mediators by macrophages (52). Recent work has also demonstrated that neutrophil microparticles carry the precursors for the biosynthesis of proresolving mediators (1, 52) that can be donated to macrophages where they are transformed to bioactive mediators (52). On-going work aims at elucidating whether microparticles are important effectors of some of these responses and the gene modulation we report here for HUVEC goes along this exciting possibility.

In conclusion, we report several novel features of neutrophil microparticles. We show that their production is a tightly regulated process with their proteomic content varying greatly depending on the nature of the stimulus. Moreover, we show that these microstructures carry distinct functional properties with an ability to synthesize inflammatory mediators such as LTB₄ and to respond to the surrounding milieu through ROS production and chemotaxis, opening novel research avenues into effector roles of microparticles in inflammatory responses. In addition, we show that the two microparticle subsets investigated during this study induced profound and distinct changes in gene expression profiles in recipient cells. These observations shed light on an area of biology that, to date, has been very little explored, highlighting the importance of a more systematic analysis of neutrophil microparticles, especially in disease. Such an analysis could provide us with robust biomarkers in disease and, equally important, the ability to determine the efficacy of specific treatment regimes, paving the way for the development of tailor-made medicines.

Acknowledgments—We thank Dr. Jayachandran Radhakrishnan and Dr. Julian Knight and the Genomic Advances in Sepsis (GaInS) Investigators for providing us with the CAP samples and Dr. Nicola Ambrose for assistance in collecting the blister exudates. We would also like to thank Dr. Charles Mein (William Harvey Research Institute, Genome Centre) for assistance with the Microarray analysis and Dr. G Wames (Blizard Institute of Cell and Molecular Sciences, Flow Cytometry Core Facility) for expert assistance with the flow cytometry experiments.

This work was supported by The William Harvey Research Foundation, The Wellcome Trust (program 086867/Z/08) and, partly, the Intensive Care Society. This work forms part of the research themes contributing to the translational research portfolio of Barts and the London Cardiovascular Biomedical Research Unit, which is supported and funded by the National Institutes of Health Research (NIHR). The research was supported in part by NIHR Biomedical Research Centre based at Guy’s and St Thomas’ NHS Foundation Trust and King’s College London in partnership with King’s College Hospital. M.M. is a Senior Fellow of the British Heart Foundation.

This article contains supplemental Figs. S1 and S2 and Tables S1 to S4.

‡‡ The authors contributed to this work equally.

** To whom correspondence should be addressed: The William Harvey Research Institute, Barts and The London Medical School, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, United Kingdom. Tel.: +44-2078828782; Fax: +44-207-8826076; E-mail: m.perretti@qmul.ac.uk.

REFERENCES

1. Norling, L. V., Spite, M., Yang, R., Flower, R. J., Perretti, M., and Serhan, C. N. (2011) Cutting edge: Humanized nano-proresolving medicines mimic inflammation-resolution and enhance wound healing. J. Immunol. 186, 5543–5547

2. Kunder, C. A., St John, A. L., Li, G., Leong, K. W., Berwin, B., Staats, H. F., and Abraham, S. N. (2009) Mast cell-derived particles deliver peripheral signals to remote lymph nodes. J. Exp. Med. 206, 2455–2467

3. Lynch, S. F., and Ludlam, C. A. (2007) Plasma microparticles and vascular disorders. Br. J. Haematol. 137, 38–48

4. Beyer, C., and Pisetsky, D. S. (2010) The role of microparticles in the pathogenesis of rheumatic diseases. Nat. Rev. Rheumatol. 6, 21–29

5. Wang, G. J., Liu, Y., Qin, A., Shah, S. V., Deng, Z. B., Xiang, X., Cheng, Z., Liu, C., Wang, J., Zhang, L., Grizzle, W. E., and Zhang, H. G. (2008) Thymus exosomes-like particles induce regulatory T cells. J. Immunol. 181, 5242–5248

6. Montes, M., Jaenssou, E. A., Orozco, A. F., Lewis, D. E., and Corry, D. B. (2006) A general method for bead-enhanced quantitation by flow cytometry. J. Immunol. Methods 317, 45–55

7. Tung, J. W., Parks, D. R., Moore, W. A., and Herzenberg, L. A. (2004) New approaches to fluorescence compensation and visualization of FACS data. Clin. Immunol. 110, 277–283

8. Abdullah, N. M., Kachmar, M. M., Walker, A., Hawley, A. E., Wrobleski, S. K., Myers, D. D., Strahler, J. R., Andrews, P. C., Michailidis, G. C., Ramacciotti, E., Henke, P. K., and Wakefield, T. W. (2009) Microparticle surface protein are associated with experimental venous thrombosis: a preliminary study. Clin. Appl. Thromb. Hemost. 15, 201–208

9. Peterson, D. B., Sander, T., Kaul, S., Wakim, B. T., Halligan, B., Twigger, S., Pritchard, K. A., Jr., Oldham, K. T., and Du, J. S. (2008) Comparative proteomic analysis of PAI-1 and TNF-alpha-derived endothelial microparticles. Proteomics 8, 2430–2446

10. Gasser, O., Hess, C., Miot, S., Deon, C., Sanchez, J. C., and Schiffri, J. A. (2003) Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils. Exp. Cell Res. 285, 243–257

11. Porro, C., Lepore, S., Trotta, T., Castellani, S., Ratclif, L., Battaglini, A., Di Gioia, S., Martinez, M. C., Conese, M., and Maffione, A. B. (2010) Isolation and characterization of microparticles in sputum from cystic fibrosis patients. Respir Res 11, 94

12. Dalil, J., Norling, L. V., Renshaw, D., Cooper, D., Leung, K. Y., and Perretti, M. (2008) Annexin 1 mediates the rapid anti-inflammatory effects of neutrophil-derived microparticles. Blood 112, 2512–2519

13. Distler, J. H., Pisetsky, D. S., Huber, L. C., Käldén, J. R., Gay, S., and Distler, O. (2005) Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases. Arthritis Rheumatism 52, 3337–3348

14. Rautou, P. E., Vion, A. C., Amabile, N., Chironi, G., Simon, A., Tedgui, A., and Boulanger, C. M. (2011) Microparticles, vascular function, and atherothrombosis. Circulation Res. 109, 593–606

15. Angelillo-Scherrer, A. (2012) Leukocyte-derived microparticles in vascular homeostasis. Circulation Res. 110, 356–369

16. Mostefai, H. A., Meziani, F., Mastronardi, M. L., Agouni, A., Heymes, C., Sargentini, C., Asfar, P., Martinez, M. C., and Andriansitohaina, R. (2008) Circulating microparticles from patients with septic shock exert protective role in vascular function. Am. J. Respir. Crit. Care Med. 178, 1148–1155

17. Tamagawa-Mineoka, R., Katoh, N., and Kishimoto, S. (2010) Platelet activation in patients with psoriasis: increased plasma levels of platelet-derived microparticles and soluble P-selectin. J. Am. Acad. Dermatol. 62, 621–626

18. Guiducci, S., Distler, J. H., Jungel, A., Huscher, D., Huber, L. C., Michel, B. A., Gay, R. E., Pisetsky, D. S., Gay, S., Matteucci-Cerinic, M., and Distler, O. (2008) The relationship between plasma microparticles and disease manifestations in patients with systemic sclerosis. Arthritis Rheumatism 58, 2845–2853

19. Prokopi, M., Pula, G., Mayr, U., Devue, C., Gallagher, J., Xiao, Q., Boulanger, C. M., Westwood, N., Urbich, C., Willett, J., Steiner, M., Breuss, J., Xu, Q., Kiechl, S., and Mayr, M. (2009) Proteomic analysis reveals presence of platelet microparticles in endothelial progenitor cell cultures.
null