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Application of Flow Cytometry in the Studies of Microparticles

Monika Baj-Krzyworzeka, Jarek Baran, Rafał Szatanek and Maciej Siedlar
Department of Clinical Immunology, Jagiellonian University Medical College, Cracow, Poland

1. Introduction

Many cell types including leukocytes, platelets and endothelial cells release small membrane fragments called microparticles (MP). MP are shed during cell growth, activation, proliferation, senescence and apoptosis. MP contain proteins (intracellular as well as surface markers), mRNA and miRNA of the cells they have originated from. Based on the release mechanism, size and phenotype, MP are frequently divided into two categories: exosomes and ectosomes called also microvesicles. There is no doubt that the biological significance of MP has been largely overlooked for many years, regarding them as merely cellular fragments or debris. Nowadays, MP are being recognized as an important regulator of cellular interactions under physiological and pathological conditions. MP are present in all body fluids and physiologically serve various functions like blood clotting, enhance cell adhesiveness, increase cell aggregation, etc. They mediate cell-to-cell communication by transferring cell surface receptors, mRNA, and miRNA from the cell of origin to target cells. The growing body of literature regarding the role of MP in many pathologies has recently progressed from describing the association of elevated MP number with disease stage (e.g. cancer, sepsis) through understanding how MP may contribute to thrombosis, preeclampsia and tumor progression, and finally, to using MP as a source of antigens in new forms of vaccines against infectious or malignant diseases.

Flow cytometry is a preferred method in the studies of MP because of its ability to quantitate the absolute number of particles and multicolor analysis attributes, allowing detection of several markers simultaneously. However, despite its usefulness, flow cytometry has some limitations in this field. The definition of MP using flow cytometry is still an area of great debate. In this review we propose a comprehensive summary of the possibilities, advantages and disadvantages of flow cytometry as a "gold standard" in the studies of MP.

2. Overview of different forms of microparticles

2.1 Definition of various MP – plenty is a plaque

MP are defined as a mixture of heterogeneous vesicles size-wise, and there is a number of schemes trying to classify them by considering their different characteristics (i.e. origin, size, distinct cell surface and/or internal determinant patterns, etc.), which may become confusing at times.

One of the most routinely used schemes in defining MP is their cellular origin [Heijnen et al., 1999, Hess et al., 1999, Dumaswala et al., 1984, Ginestra et al., 1998, Zitvogel et al., 1998]. This method utilizes flow cytometry to compare the cell membrane determinant composition as well as the internal cargo of MP with that of the original cell. Scientists that chose this method do not restrict themselves to just one surface/cellular determinant, but use many, in order to define the MP most precisely. It is very desirable in this case to have a unique determinant (present on/in the original cell and its MP) that would definitely establish the MP origin. Based on this classification method, scientists then use terminology that stresses out the MP origin, i.e. dendritic cell-derived microvesicles, erythrocyte-derived microvesicles, platelet-derived microvesicles, etc. [Heijnen et al., 1999, Hess et al., 1999, Dumaswala et al., 1984, Ginestra et al., 1998, Zitvogel et al., 1998].

Another way of defining MP considers two populations of MP, ectosomes and exosomes, depending on the place of origin within the same cell [Pilzer et al., 2005, Rak et al., 2010]. Thus, ectosomes are considered to be vesicles which are formed upon plasma membrane vesiculation, where as exosomes are generated within endosomal structures inside a cell [Al-Nedawi et al., 2009]. Ectosomes are relatively larger than exosomes with their size ranging from 100-1000 nm in diameter, and their outer membrane is rich in phosphatidylserine (PS) residues [Al-Nedawi et al., 2009, Del Conde et al., 2005]. Ectosomes are also associated with the formation of lipid rafts, membrane regions containing high levels of cholesterol and signaling complexes. Exosomes, on the other hand, have a lower abundance of PS residues compared to ectosomes and are usually smaller (30-100 nm) in diameter [Simpson et al., 2009]. They also seem to transport a different type of cargo than other MV originating from the same cell, showing more transporting selectivity for intracellular proteins/molecules.

Another group of MP that is often regarded as a separate population is derived from tumor cells. The most important criterion in defining TMV seems to be the assessment of their tumor origin [Yu et al., 2005, Bergmann et al., 2009, Kim et al., 2003, Huber et al., 2005]. Here, again, flow cytometry is used as the means of establishing the determinant composition (surface and/or internal) of TMV which is then compared to that of the tumor cells.

Considering the different ways of MP classification it has to be kept in mind that there is no clear, well-defined approach that could be applied for MP differentiation. Many of the methods mentioned above tend to overlap, which when used separately, could result in defining the same MP population. There is a growing need of trying to establish clear-cut guidelines that would help properly define the different types of MP. Figure 1 is a representation of the heterogeneity of MP and their different nomenclature. It also depicts some possible determinants that can be transported by particular MP population.

2.2 Isolation of MP from biological fluids or culture supernatants – The devil is in the details

There is no real consensus or a uniform protocol on MP isolation from different types of biological fluids or culture supernatants. Most of the time, people who try to isolate MP develop their own protocols which tend to incorporate their particular interests as well as the availability of equipment in their laboratory setting. The other side to this problem is

sample preparation such as collecting, processing temperature, etc. as well as how the samples should be stored and prepared for future use.

The most commonly used methodology for MP isolation is differential centrifugation which employs a number of different centrifugation steps characterized by different centrifugal forces and centrifugation times. The concept behind this type of methodology is to purify the sample in such a manner as to obtain the correct MP population. Although, differences between the protocols exist, there seems to be a general agreement on the purification of the MP sample from cells and other larger cellular fragments that remain after a sample collection. This is regardless of the sample origin, whether it is culture supernatants, plasma or any other biological fluid. Thus, the initial purification step is set at a relatively low centrifugal force (around 200-500xg) for a short time period (app. 10 min.) to get rid of the larger fragments/cells from the sample [Orozco et al., 2010, Baran et al., 2010]. The next step, which centrifugal force ranges between 10,000-17,000xg and is set between 30 min. to 1 hour, is designed either to obtain the MP or to further purify the sample of unwanted cellular fragments or MP [Ayers et al., 2011, Baran et al., 2010, Gelderman & Simak, 2008]. One has to consider its interest because at this speed some of the MP can be lost which might be of significance. Thus, if platelet-derived MP (PMP), whose presence is predominant in plasma samples, is a subject of the study then the pellet obtained after this centrifugation step will mainly consist of them. At this step, one has to also consider the impact of size with regards to MP because if exosomes (part of MP population), which are considered to be smaller vesicles, are the subject of the study then they will remain in the supernatant, and the next centrifugation step/steps is/are considered to be crucial to obtain them (centrifugal force up to 150,000xg) [Grant et al., 2011, Baran et al., 2010, Al-Nedawi et al., 2008].

The adopted form of verifying the individual steps of the different centrifugation protocols employs flow cytometry. Staining the pellet or supernatant samples obtained during the different phases of the protocol with appropriate antibodies and then analyzing them using flow cytometry seems to be the method of choice by many groups. The idea behind it is to trace the MP fractions, whether present in the pellets or supernatants that come off during each step of the centrifugation protocol. Thus, for example, if platelet-free sample is required, one would stain the pellet and supernatant obtained after the initial and the 10,000-17,000xg centrifugation steps to check for platelet markers (i.e. CD41, CD61) [Baran et al., 2010]. The depletion of $CD41^+$ or $CD61^+$ entities would then signal that sample purification was successful and that further sample processing can be initiated (Fig. 2 represents an example of a marker tracing (CD61) on MP in the supernatant fractions of a plasma sample obtained during different centrifugation steps). Analogically, flow cytometry is used when the final MP population is obtained and needs verification or characterization with respect to the surface determinant profile.

Another form of centrifugation, which is also commonly used in MP isolation, involves the application of a sucrose gradient [Lamparski et al., 2002, Keller et al., 2011, Zhong et al., 2011]. In the case of the sucrose gradient filtration method, a sucrose gradient is created by gently overlaying a sucrose layer of lesser concentration on top of the layer with higher sucrose concentration in some defined concentration increments in an appropriate test tube. Next, the sample is placed on top of the sucrose density gradient and subjected to a high centrifugal force (app. 150,000xg) for an extended time period. During the centrifugation step, the sample particles move through the gradient until they reach the sucrose density

that matches their own, where they stop. At the end of this step, the obtained fractions that contain MP according to their different densities, can be removed and utilized in further tests.

Another method for isolating MP which is gaining interest is referred to as microfluidic immunoaffinity method [Chen et al., 2010, Hsu et al., 2008]. The propagators of this procedure point out that this method is much faster in comparison to differential centrifugation or sucrose gradient filtration and that it yields higher MP recovery rates [Chen et al., 2010]. Another major advantage to this method seems to point out to the fact that smaller sample volumes are being used as well as the isolated MP remain relatively "untouched" (the impact of centrifugal force being excluded) thus resembling more accurately their native state i.e. shape, determinant surface profile, etc. The principle behind this method is to selectively bind MP populations to antibody-coated surfaces [Chen et al., 2010, Hsu et al., 2008, Choi et al., 2011, Cheng et al., 2007] . The method utilizes a flow channel of different dimensions, depending on the initial sample volume to be processed, which surface is chemically modified in order to later coat it with appropriate antibodies. The outcome of such modification results in the preparation of a column that is coated with an antibody that recognizes a surface determinant characteristic for particular MP population. Next, a sample is injected into the channel and the appropriate MP are immobilized on its surface. This is followed by a washing step which purpose is to elute the immobilized MP which then can be used for further tests (protein analysis, RNA isolation, etc.).

Another aspect of MP isolation that has a major impact on MP quality is sample collection, preparation and storage [Dey-Hazra et al., 2010, Trummer et al., 2009, Shah et al., 2008]. Unfortunately, here again no uniform protocols exist that would state the proper way to address these issues. As with different isolation protocols, the sample collection, preparation and/or storage depend on individual needs and settings characteristic of the study. However, there seems to be a general understanding that freshly obtained samples are the best to work with and that multiple "freezing and thawing" has a substantial impact on both the MP number as well as the surface determinant expression, which suggest that, if possible, should be avoided (see section 2.1.1) [Dey-Hazra et al., 2010, Trummer et al., 2009, Shah et al., 2008].

2.3 Sizing of MP and estimation of their absolute number by flow cytometry – Role of instrumentation

Flow cytometric analysis of MP appears to be the most favored method used for their characterization [Jy et al. 2004]. Typically, MP are identified as particles with a forward scatter smaller than an internal standard consisting of 1-1.5 μm sized latex particles [Shet et al., 2003]. Light scattering is a basic phenomenon for detecting and characterizing particles in modern flow cytometry. A light beam directed at a particle can interact with it through reflective, refractive and diffractive effects. Then, information about a particle or aggregates of particles can be derived from the changes in direction and intensity of the scattered light [Kim & Ligler 2010]. Collecting scattered light at various angles from the incident beam has been reported to provide different types of information about the particle, including both size and density [Shvalov et al., 1999]. Typically, forward scattered light (0.5-5°) can provide approximate information about the size of particle [Shapiro 2004]. It should, however, be

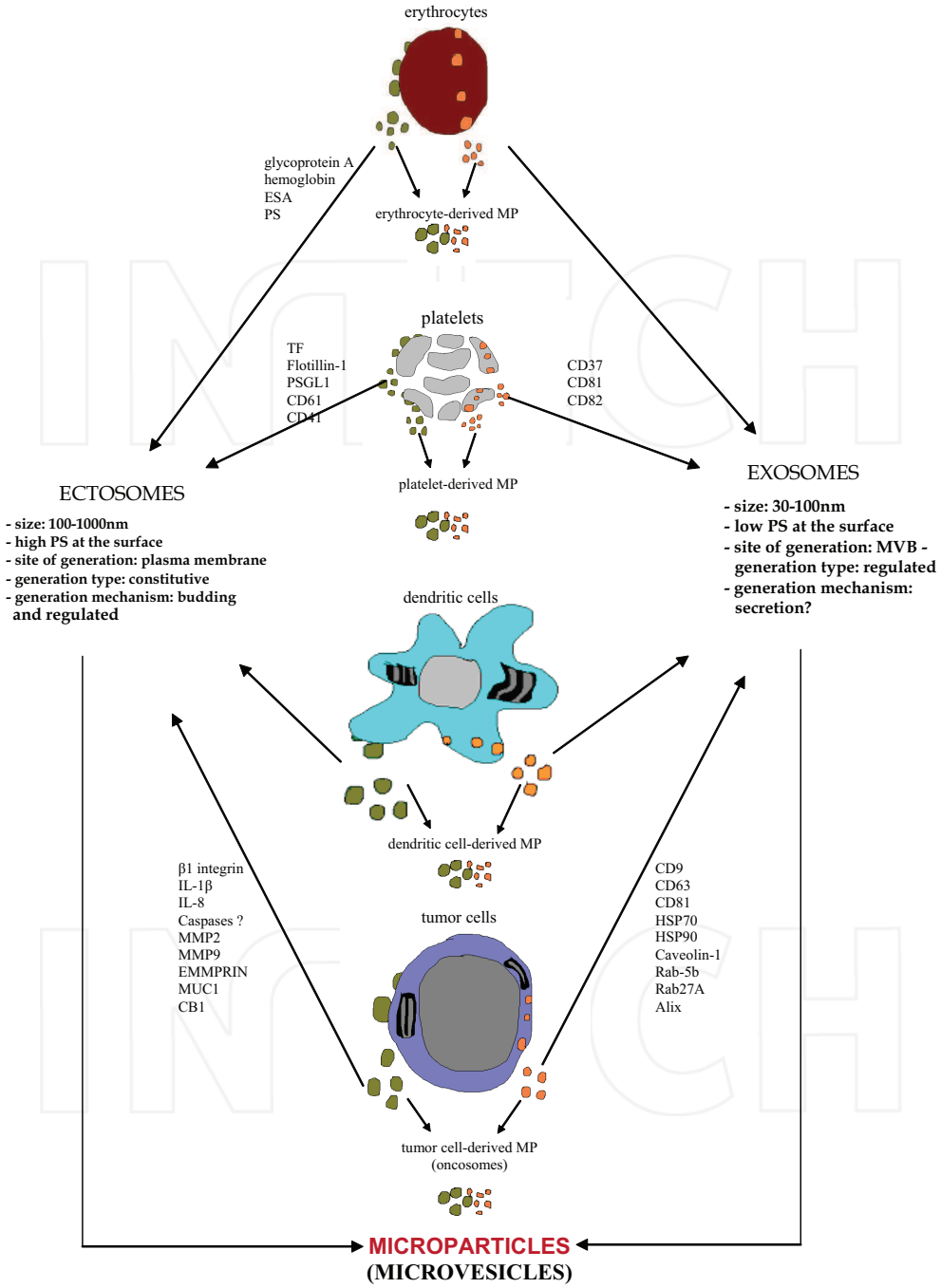


Fig. 1. Heterogeneity of microparticles

noted that the intensity of FSC is not related directly to particle size [Becker et al., 2002]. On the other hand, side-scattered light (15-150°) is often collected at 90° angle and provides information about smaller particles or granularity of internal structures. Measuring side-scattered and forward scattered light has become a standard in biomedical research, enabling cells to be distinguished by size and granularity.

A problem with MP in a standard flow cytometry is that they cannot reliably be detected when setting a forward scatter as a trigger, as they are smaller than the wavelength of a laser light used, and cannot always be discriminated from the background noise. Moreover, forward scatter is the most variable signal between instruments of different manufacturers and its proper alignment is crucial. It is affected by refractive index mismatches between sheath fluid and sample, beam geometry, polarization, beam stop position and collection angle [Nebe-von-Caron, 2009]. Unfortunately, there are a lot of data published in peer reviewed journals based on the assumption that a forward scatter signal of certain size beads represents similar size in MP. The problems with this approach have been already highlighted, and nowadays it is recommended to use log side scatter for comparative analysis of MP and beads, as all the instruments show good correlation with regards to side scatter response, being capable of reproducing the same level of sensitivity against the particular 190 nm latex particles [Nebe-von-Caron, 2009]. While the identification of MP on the basis of the light scattered parameters tests the limit of sensitivity of flow cytometry, some investigators have overcome this problem by setting the parameters of the instrument to detect fluorescence as a trigger (Horstman et al, 1994). Thus while analyzing MP one should look at log side scatter versus log fluorescence of the selected staining triggered on fluorescence and side scatter at the instrument noise level (if triggered on two channels) or either of the two, depending on the analysis needed [Nebe-von-Caron 2011].

Enumeration of MP by flow cytometry

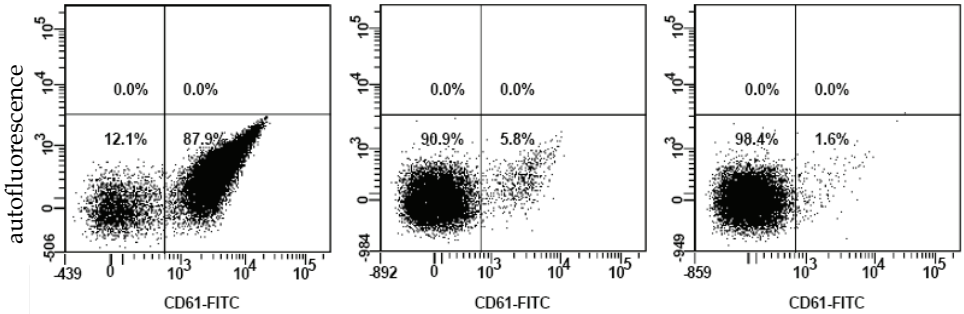
Flow cytometry can also be used to enumerate MP (usually in the plasma) by adding, as an internal standard, a known number of fluorescent latex beads (Flow-Count Fluorospheres, Beckman-Coulter) or using tubes containing already predefined number of them (TruCount tubes, BD Biosciences). The number of MP present in the sample is derived from the following formula (1) and adjusted for the final dilution of the original sample [Baran et al, 2010]:

$$\text{MP count} = \frac{\text{No. of events in region containing MP}}{\text{No. of events in absolute count bead region}} \times \frac{\text{No. of beads per test}}{\text{Test volume [}\mu\text{l]}} \quad (1)$$

Alternatively, if the flow cytometry instrument delivers the sample to the optics by screw-driven syringe at a known rate, then the sample MP count can be calculated [Orozco & Lewis, 2010].

Role of instrumentation

Recently, a class of flow cytometers has been developed which allows the detection of particles of down to 100 nm in size. The developers of these cytometers state that the unique optical design of the apparatus eliminates the unwanted light thus giving the best signal to noise ratio. Due to these adjustments, the cytometers are supposedly representing the highest light scatter sensitivity and resolution available making it an attractive tool in MP research. Additionally, they also offer the volumetric-based absolute count ability of



(A) (B) (C)

Fig. 2. Tracking of CD61 expression to follow platelets and PMP elimination in a gastric patient plasma sample subjected to differential centrifugation. A- initial plasma; B- plasma subjected to centrifugation at 15,000xg; C- plasma subjected to centrifugation at 50,000xg

samples as well as high fluorescence sensitivity making their use even more practical. One of such product is manufactured by Apogee Flow Systems and allows detection of particles less than 150 nm using 3 light scatter detectors. However, for appropriate MP detection an even lower detection limit is required as compared to microspheres [Chandler, et al. 2011]. For smaller particles, like exosomes (< 100 nm in diameter) some other approaches can be introduced to allow their analysis by flow cytometry. Caby et al. in a very elegant study have presented detection and characterization of peripheral blood exosomes by flow cytometry analysis using CD63-coated latex beads, which can be easily detected by standard instrumentation [Caby et al., 2005]. Other approaches introduce electrical detection systems to detect beads with attached MP/exosomes. Those are based on the work of Wallace Coulter, who demonstrated that electrical charge can be used to detect, size and count particles in solution. Advances in nanotechnologies have drawn many research groups to develop Coulter counters on chip-based platforms [Kim & Ligler 2010]. Holmes et al. has demonstrated a microfabricated flow cytometer for rapid analysis of microspheres using impedance for particle detection [Holmes et al., 2007]. Such cytometers could measure impedance at high (10 MHz) and low (0.5 MHz) frequencies to distinguish mixed bead populations [Kim & Ligler, 2010]. These beads may be coated with antibodies capturing MP/exosomes of different origin.

2.4 Multicolor flow cytometry analysis of MP

Multicolor flow cytometry analysis of MP, using monoclonal antibodies, opened a new way of extensive investigation and characterization of MP. Multicolor analysis is used to detect the cellular origin of MP based on their phenotype. Gelderman and Simak have developed a three-color flow cytometric assay for immunophenotyping MP that are present in plasma [Gelderman & Simak, 2008]. The assay has been used to study MP present in plasma of healthy donors and in patients with paroxysmal nocturnal hemoglobinuria, sickle cell anemia, and in patients with acute ischemic stroke [Simak et al., 2002, Simak et al., 2004, Simak et al., 2002a]. With the use of monoclonal antibodies conjugated to different fluorochromes, a combination of three or even more antigens can be analyzed on a single particle. In addition, annexin V conjugated to a fluorochrome can be used to detect PS on

MP. Some authors limit the analysis of MP to only PS positive ones, however, it has been shown that only a limited population of MP in blood binds annexin V. Although immunophenotyping of MP seems to be a straightforward procedure, here are some methodological requirements in MP staining with monoclonal antibodies. First of all, monoclonal antibodies should be directly conjugated to fluorochomes (indirect staining is not recommended), and fluorochromes used should be as bright as possible (FITC – fluorescein isothiocyanate and PerCP – peridinin-chlorophyll-protein complex conjugated antibodies are not recommended). The titration of antibodies using MP prepared from their parental cells *in vitro* as well as from plasma, should be a rule. Gelderman and Simak recommend the use of two clones against different epitopes of an antigen to confirm specificity of detection [Gelderman & Simak, 2008]. In regard to identification of cellular origin, they suggest using glycophorin A (CD235a) and the leukocyte common antigen (CD45) for detection of red blood cell and leukocyte derived MP, respectively. Platelet-derived MP are detected using monoclonal antibodies against GPIIb (CD41) or GPIIIa (CD61). Monoclonal antibodies to CD14, CD66b, CD4, CD8 and CD20 are used to detect MP from monocytes, granulocytes, T helper, T suppressor and B lymphocytes, respectively. For endothelial-derived MP the use of anti-PECAM (CD31), anti-CD34, anti-E selectin (CD62E) and anti-Endoglin (CD105) monoclonal antibodies is recommended [Gelderman & Simak 2008]. And finally, relevant isotype controls to detect the nonspecific staining should be used in parallel. It is of importance to keep in mind that the presence of specified antigen on MP does not clearly identify their cellular origin. In some cases MP may absorb a soluble antigen circulating in the plasma that is derived from another cell type. A good example is absorption of prostate antigen (PSA) by human monocytes [Faldon et al., 1996]. Thus MP derived from such monocytes might be positive for PSA.

3. Biology of platelet derived microvesicles (PMP)

PMP are the most abundant MP population in blood stream constituting approximately 70-90% of circulating MP [Horstman et al., 1999]. First demonstration of "platelet dust" was done in 1967 by Wolf [Wolf, 1967]. Nowadays, it is known that PMP may be released by activated as well as resting platelets, both in circulation and *in vitro* experiments. A body of experimental and clinical data has shown the association between PMP and diseases.

3.1 Characteristics of PMP

3.1.1 Methods of PMP generation and their phenotype analysis by flow cytometry

Blood platelets activated by a variety of stimuli undergo shape change and degranulation. During this process platelets secrete thin walled vesicles called microparticles or microvesicles (PMP) and smaller exosomes. Under physiological conditions PMP are released after activation with agonists such as epinephrine, adenosine diphosphate, thrombin or collagen [Matzdorff et al., 1998], and also by exposure to complement protein C5b-9 or high shear stress (higher than physiological), [Holme et al., 1997]. *In vitro*, PMP may be generated by stimulation of platelets with physiological factors like thrombin [George, 1982], thrombin and collagen [Baj-Krzyworzeka et al., 2002], or nonphysiological agonist like calcium ionophore [Forlow et al., 2000]. PMP shedding was also described in stored and apoptotic platelets [Jy et al., 1995].

Preanalytical stages like blood sampling site (cubital vein, central venous caterer), needle diameter, discharge of the first portion of blood, collection (Vacutainer, tube, etc), anticoagulant and transport circumstances are extremely important for PMP quality and quantity. Blood should be anticoagulated with 3.8% sodium citrate (9:1 v/v, BD Vacutainer blood collection tubes are recommended) as versenate salts lead to platelet aggregation. Changes in temperature, like overheating or cooling should be avoided. Also, shaking of blood may induce PMP release from platelets as well as other blood cells. The delay between sampling and further processing should be as short as possible (less than 1 hour), as storage results in increased number of PMP [Simak & Gelderman, 2006, Kim et al., 2002].

The protocols for obtaining PMP differ between laboratories and their standardization is a subject of debate. The first step in standardization was undertaken by the Scientific Standardization Committee of the International Society on Thrombosis and Haemostasis. The Committee recommends double centrifugation step to ensure removal of platelets from platelet poor plasma (PPP) and washing PPP samples to remove non specific particles, however these procedures may results in the loss of MP [Ayers et al., 2011]. Differential centrifugation-protocol results in isolation of PMP that in many cases contain exosomes. To avoid the exosomes contamination Grant et al. proposed filtration of PPP through a 0.1 μm pore size filter [Grant et al., 2011]. MP may be also isolated from PPP by capturing them using immobilized annexin V which binds to PS present on PMP and other MP. In this assay, MP are captured with biotinylated annexin V, then incubated with streptavidin-coated plates, and after being washed used for further experiments [Mallat et al.,1999].

Flow cytometry in PMP analysis

Flow cytometry allows the analysis of large numbers of PMP using tiny volume (about 5 μl) of plasma [Simak & Gelderman, 2006, Michelson et al., 2000]. It is of importance to use double filtered (0.2 μm) sheath fluid (e.g. phosphate buffered saline - PBS) for flow cytometry analysis of PMP. Generally, an accepted background "noise" consists of 25-50 events per second when filtered PBS is run.

Size. Standard beads of different sizes (diameter) may, to some extend, be used for PMP sizing, e.g. monodisperse fluorescent Megamix beads (BioCytex). However, one has to be aware that size related data derived from beads and that derived from biological particles are not fully comparable [Lacroix et al., 2010].

It is generally accepted that PMP are larger than 100 nm, but at the same time it should be noted that PMP are very heterogeneous size-wise. The upper limit of PMP size is about 1.5 μm. Additionally, distinguishing between PMP aggregates and platelet-PMP aggregates by flow cytometry may cause problems [Simak & Gelderman, 2006], because of their size overlap. Better results in platelet/PMP sizing can be achieved by using impedance-based flow cytometry instead of light-scatter based one. For this purpose electron microscopy or AFM analysis are highly recommended [Yuana et al., 2011].

Number. App. 75% of laboratories use flow cytometry to enumerate PMP in clinical samples [Lacroix et al., 2010]. However, a variety of preanalytic and analytic variables may lead to variations in PMP values in healthy individuals ranging from 100 to 4000×10^3 per μl [Robert at al., 2009]. Although, standardization in PMP count is still inadequate some steps

have been already undertaken to uniform this procedure. Three Scientific and Standardization Subcommittees of the International Society on Thrombosis and Haemostasis (ISTH) have initiated a project aimed at standardizing an enumeration of cellular MP by flow cytometry. The main objective was to establish the resolution and the level of background noise of the instruments, and to define reproducibility of PMP count in plasma using flow cytometers manufactured by different vendors. The study demonstrated that different systems were heterogeneous with respect to FSC resolution and background noise. In 2010 the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis proposed procedures to be followed for obtaining good reproducibility in PMP count by flow cytometry [Lacroix et al. 2010]. The advised strategy is based on the use of size-calibrated fluorescent beads (0.5 µm and 0.9 µm) in a fixed ratio (Megamix) to gate PMP in a defined size-restricted window [Robert et al. 2009]. However, the procedure seems to be more adequate for Beckman-Coulter instruments rather than Becton-Dickinson's, as the former measure the forward-scattered light at a relatively wide angle (1-19°) compared to the latter (1-8°) [Lacroix et al. 2010].

Phenotype. Flow cytometry is used as a method of choice for PMP phenotyping. Similarly to platelets, almost all PMP, express glycoprotein complex IIb-IIIa (CD41/CD61) [Heijnen et al., 1999, George, 1982], CD42a [Simak and Gelderman, 2006, Matsumoto et al., 2004]. Caution is recommended when staining for glycoprotein Ib (CD42b) as only 40-50% of PMP express this marker. Usually, large PMP stain better than the small ones [Zdebska et al., 1998]. Other markers were also detected on PMP, such as: CD31, PAR1, CXCR4, CD154, PF4, as well as ligands for annexin V or lactadherin [Ayers et al., 2011, Baj-Krzyworzeka et al., 2002, Fig 3.]. It has been suggested that CD41$^+$ and CD42$^+$ PMP represent two different populations, thus it is important to use both of these markers for better assessment. PMP released by activated platelets express also platelet activation markers such as CD62P and activated complex GPIIb-IIIA [Michelson, 2000]. Flaumenhaft et al. showed that cultured megakariocytes shed MP positive for CD41 and concluded that a part of blood CD41$^+$ MP

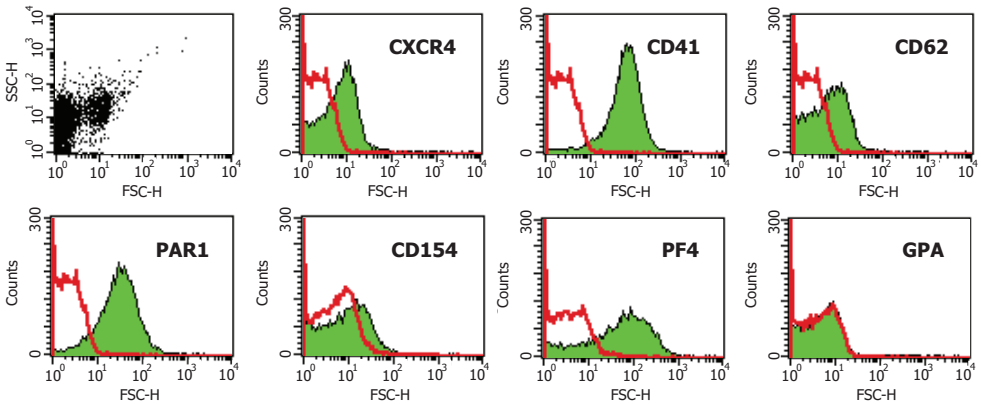


Fig. 3. Expression of selected surface proteins on PMP obtained from platelets stimulated by calcium ionophore A23187. Staining with an isotype control is shown as a red line. GPA-glycophorin A (negative marker)

are of megakaryocyte origin [Flaumenthaft et al., 2009, 2010]. They recommended multicolor flow cytometric analysis (CD41, CD62P and LAMP-1) to distinguish PMP from megacaryocyte-derived MP [Flaumenthaft et al., 2009, 2010]. Flow cytometry analysis of PMP needs standardization comprising consensual panel of antibodies (clones, concentration) and beads specification. It appears that labeling low amounts of PMP results in higher variability of MFI (Mean Fluorescence Intensity) in comparison to labeling higher amounts of PMP [Orozco & Lewis, 2010]. Moreover, it should be noted that differences in *in vitro* research protocols for PMP generation result in variable PMP subpopulations which differ in size, marker expression, protein content and thrombogenic potential [Baj-Krzyworzeka et al., 2002, Dean et al., 2009, Sandberg et al., 1985].

3.1.2 Role of PMP in modulation of biological activity of human cells

Interactions of cells with PMP may result in modulation of biological function in the following ways:

- First, PMP may provide interactions between cells without the need for direct cell-to-cell contact.
  For instance, PMP activate and stimulate neutrophils to secrete proteases, may induce the transformation of peripheral blood monocytes into endothelial progenitor cells [Prokopi et al., 2009], and may facilitate the interactions between leukocytes and endothelial cells [Forlow et al., 2000, Barry et al., 1998]. PMP may also interact with $CD34^+$ cells and increase their bone marrow homing [Janowska-Wieczorek et al., 2001].
- Second, PMP can bind to target cells and fuse with their membranes, resulting in the acquisition of new surface antigens and thus new biological properties and activities of the target cells. For example, the chemokine receptor, CXCR4 which is present on PMP, may be transferred to various cells and make them susceptible to infection by X4-HIV [Rozmysłowicz et al., 2003]. PMP may also transfer GPIIb/IIIa to neutrophils (allowing its interaction with CD18) and neutrophil activation [Salanova et al., 2007] or transfer CD40L which may activate B cells [Sprague et al., 2008].
- Third, PMP may act as "transfer vehicles" which may deliver/exchange protein, lipids, mRNA or even pathogens to target cells. For instance, PMP may change cell activity by lipid transfer [Barry et al., 1997] or may deliver CCL23, CXCL4,7, FGF and TGF [Dean et al., 2009], thus transferring chemoattractant capabilities to various cells [Baj-Krzyworzeka et al., 2002, Fig. 4.]

3.2 PMP in health and disease (counting and procoagulant activity)

Changes in circulating PMP level have been described in patient with many disease states.

Hemostasis and thrombosis

The PMP number is decreased in preterm neonates compared with adults [Rajasekhar et al., 1994]. Patients with Castaman's defect and Scott syndrome, whose platelets are defective, also have a defect in generation of PMP [Castaman et al., 1996, Weiss et al., 1994], which is associated with a bleeding tendency. On the other hand, an increased number of PMP suggests a potential prognostic marker for arherosclerotic vascular disease [Boulanger et al., 2006, Michelsen et al., 2008], transient ischemic attacks, cardiopulmonary bypass and

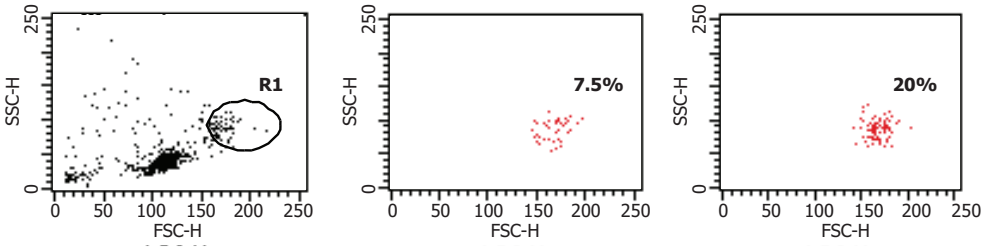


Fig. 4. Chemotactic activity of human blood monocytes to PMP analyzed by flow cytometry. The dot-plots show: monocytes defined in PBMC population by FSC and SSC parameters – region R1 (left); spontaneous chemotaxis of monocytes to culture medium without serum (middle) and chemotaxsis of monocytes to PMP (right)

thrombocytopenias (heparin-induced, thrombotic and idiopathic thrombocytopenic purpura) [Sheriadan et al., 1986, Warkentin et al., 1994]. PMP are strongly procoagulant as they contain the anionic phospholipid PS. Procoagulant PMP are present *in vivo* in blood of patients with activated coagulation and fibrinolysis including patients suffering from Disseminated Intravascular Coagulation (DIC), however, such PMP are also found in healthy donors but in low amounts only [Holme et al., 1994]. An elevated number of PMP was described in patients with diabetes mellitus, which causes the development and progression of artherosclerosis in these patients [Nomura et al., 1995].

Cancer

Although there is no set standard for monitoring PMP levels, some studies have shown that their number is elevated in the plasma of cancer patients as compared to the normal samples suggesting that the tumor itself may be responsible for stimulating their release presumably to enhance its survival. For example, elevated numbers of PMP were detected in plasma of patients with gastric cancer or in urine of patients with bladder cancer [Kim et al., 2003]. Microvesicles derived from activated platelets was also reported to induce metastasis and angiogenesis in lung cancer [Janowska-Wieczorek et al., 2005].

Inflammatory diseases

Boilard et al. demonstrated that platelets are crucial for the development of arthritis. They showed that PMP may facilitate arthritis progression [Boilard et al., 2010]. Other authors reported increased level of PMP in septic patients [Nieuwland et al., 2000, Mostefai et al., 2008]. Suprisingly, early elevated level of PMP and endothelial MP may predict a more favorable outcome in severe sepsis [Soriano et al., 2005].

4. Blood leukocyte derived MP

Major part (~80%) of MP present/detected in serum/blood is released by platelets, while the remaining ~20% constitute MP shed by erythrocytes, leukocytes and endothelial cells [Ratajczak et al., 2006].

Leukocyte-derived MP circulate in the blood stream under normal conditions and are rapidly up-regulated by inflammatory stimuli, e.g. polymorphonuclear leukocytes (PMNs)

stimulated with fMLP (formyl-methionyl-leucylphenylanin), calcium ionophore, IL-8 or PMA release small vesicles very quickly (in the matter of minutes) [Hess et al., 1999, Mesri et al., 1999]. Analysis of these MP by flow cytometry showed expression of complement receptor 1 (CR1, CD35), CD66b, HLA class I, LFA-1/CD11a, Mac-1/CD11b, CD62L, CD46, CD55, CD16 and CD59 [Gasser et al., 2003]. However, PMN-MP did not express detectable amount of others PMNs markers like CD32 and CD87 [Gasser et al., 2003]. In general, PMN-MP may be distinguished from other non-PMN-MP by expression of CD66b and annexin V binding.

MP released by monocytes can be defined by CD14, CD18 and TF expression [Aharon et al., 2008]. Moreover, these microvesicles contain caspase-1 and may deliver a cell death signal to e.g. vascular smooth muscle cells [Sarkar et al., 2009]. MP generated by the human monocytic cell line U937 were positive for the co-stimulatory molecules CD80 and CD86, whereas the expression of the adhesion molecules CD11a, CD11c (the DC marker and complement receptor 4), HLA-DR and the scavenger receptor CD36 were rather low. Also, expression of HLA class II molecules such as HLA-DR, HLA-DP and HLA-DQ was lower on MdM (monocyte-derived macrophages)-derived MP than MP of mature or immature DC [Kolowos et al., 2005]. Kolowos et al observed that MP derived from LPS-stimulated MdM showed high expression of the activation marker CD71 in comparison to MP of untreated or UV-B irradiated MdM. Stimulation of another monocytic cell line (THP-1) by starvation or by endotoxin and calcium ionophore A23187 resulted in the release of MP which expressed exosomal marker Tsg 101, monocyte markers (CD18, CD14) and active tissue factor (TF) [Aharon et al., 2008]. The number of monocyte-derived MP was elevated in meningococcal sepsis and in patients with acute coronary syndromes [Nieuwland et al., 2000].

MP derived from B lymphocytes (defined by the expression of CD19) and DC have the capacity to present antigens to induce antigen-specific T-cell responses [Raposo et al., 1996, Zitvogel et al., 1998]. Dendritic cell-derived MP showed expression of HLA class I and II, as well as costimulatory molecules CD80 and CD86 [Wieckowski & Whiteside, 2006] and may present antigens to T cells [Montecalvo et al., 2008]. DC- derived exosomes were proposed to be a short range mechanism to spread alloantigen during T cell allorecognition.

Exosomes from activated T cells can mediate "activation-induced cell death" in a cell-autonomous manner, defined by the nature of the initial T cell activation events and can play central roles in both central and peripheral deletion events involved in tolerance and homeostasis [Prado et al., 2010]. On the other hand thymic cell-derived MP express several proteins that are known to be involved in leukocyte rolling on endothelial surfaces, as well as in transendothelial leukocyte migration [Turiak et al., 2011].

5. Tumor derived microvesicles – Biology and function

Tumor derived microvesicles (TMV), also called oncosomes, are released by tumor cells during their activation (Fig. 5) by different stimuli, hypoxia, irradiation, exposure to proteins from an activated complement cascade and exposure to shear stress [Ratajczak et al., 2006].

Flow cytometry is the standard method to detect and count TMV in human plasma or ascites [Baran et al., 2010]. The number of MV (mainly platelet-derived) in cancer patients is usually elevated and may correlate with distant metastasis, however, it does not correlate with the tumor size [Hejna et al., 1999, Borsig et al, 2001]. More informative is the level of MV specific for tumor cells, e.g hepatic in hepatocellular carcinoma patients which directly correlates with the tumor size [Brodsky et al., 2008]. Counting of specific TMV is possible when specific markers of tumors are known and mAb for flow cytometry are available.

TMV were reported to reprogram endothelial cells by increasing their proangiogenic activity by inducing VEGF production [Al-Nedawi K., et al., 2009; Skog J., et al. 2008]. Also, TMV containing EMMPRIN have been shown to transactivate matrix metalloproteinase (MMP) in peritumoral stromal cells thus increasing tumor spread [Siddhu et al. 2004]. It has been also suggested that polymerization of fibrin by TMV may cause an increase in the entrapment and adhesiveness of tumor cells [Castellana et al. 2010].

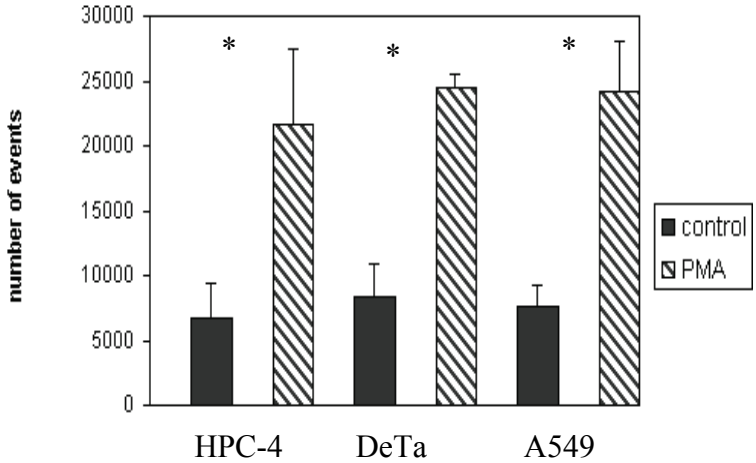


Fig. 5. Comparison of TMV number in culture supernatants from control and stimulated with PMA (100ng/ml) human cell lines: HPC-4 (pancreatic adenocarcinoma), DeTa (colorectal adenocarcinoma) and A549 (lung carcinoma). The number of events (TMV) recorded in the supernatants during 20s of acquisition using FACSCalibur. * $p<0.01$

5.1 TMV as a carrier for proteins, mRNA and miRNA (flow cytometry, Western blotting, real-time PCR)

TMV display a broad spectrum of bioactive substances and receptors on their surface. TMV from melanoma, glioma, breast, lung and pancreatic tumor cell lines express several surface molecules such as CD44, CD63, CD95L, CD147 (extracellular matrix metalloproteinase inducer, EMMPRIN), EpCAM, MUC1, oncogenic receptor EGFRvIII, integrins, chemokine receptors, TNF receptors and HLA molecules [Andreola et al., 2002, Dolo et al., 1995, Friedl et al., 1997, Fritzsching et al., 2002, Sidhu et al., 2004, Taylor and Gercel-Taylor, 2005, Al-Nedawi et al., 2008, Baj-Krzyworzeka et al., 2006]. Membrane – anchored receptors presented on tumor cell are usually present on TMV, but the level of

their expression does not always correlate with that on the original cells, e.g. CD44H expression on pancreatic adenocarcinoma cells is abundant but on their TMV rather low [Baj-Krzyworzeka et al., 2006]. At the same time, TMV expressed a higher percentage of CD44 variants (v6 and v7/8) than tumor cells [Baj-Krzyworzeka et al., 2006]. Expression of tumor markers such as Her-2/neu, c-MET, EMMPRIN and MAGE-1 was confirmed by Western blotting [Baran et al., 2010].

The complete characterization of TMV protein content is possible only by mass spectrometry-based proteomic analysis. In an elegant study, Choi et al. identified in microvesicles/exosomes derived from the plasma of colorectal cancer patients 846 proteins involved in tumor progression. [Choi et al., 2011].

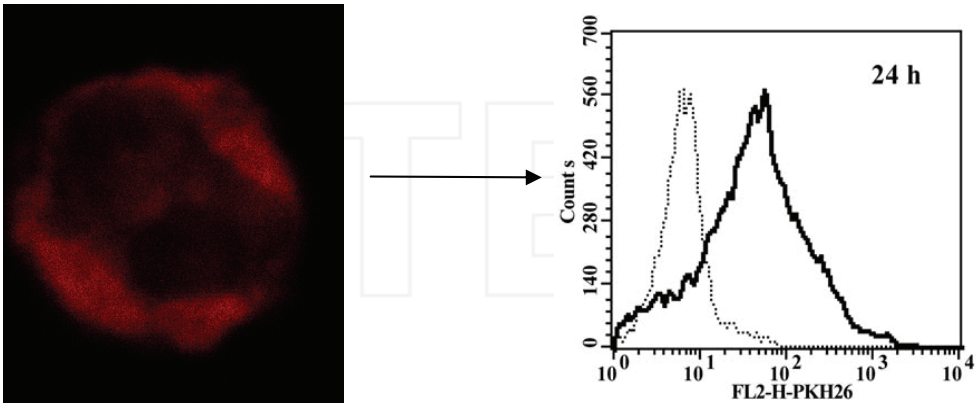
TMV contain mRNA for chemokines, growth factors, cytoskeleton proteins and tumor markers, as assessed by real-time PCR [Baj-Krzyworzeka et al., 2006, 2011, Ratajczak et al., 2006, Baran et al., 2010]. TMV may transfer mRNA and smaller RNA molecules, such as microRNA to responding cells [Ratajczak et al., 2006, Camussi et al., 2010]. Also, exosomes contain microRNAs and small RNA but very little mRNA [Zomer et al., 2010].

In conclusion MP can be considered as "macro messengers" as they can deliver proteins, mRNA and microRNA at the same time.

5.2 Tracking of fluorescently labeled TMV *in vitro* and *in vivo*

The phenomenon of protein/receptor transfer by MP was described for the first time by Mack et al. Transfer of CCR5 positive MP to T cells enable M- tropic HIV-1 infection [Mack et al. 2000]. Transfer of PMP increased adhesion of "painted" cells to fibrinogen [Baj-Krzyworzeka et al., 2002]. The same mechanism of receptor transfer was observed in the case of TMV. After a short incubation time transfer of TMV-related molecules CCR6, CD44v7/8 to monocyte was observed [Baj-Krzyworzeka et al., 2006]. Moreover, transferred receptors remained functional [Mack et al., 2000, Baj-Krzyworzeka et al., 2006]. To assess TMV location in monocytes confocal microscopy and flow cytometry analyses with extracellular fluorescence quenching were employed [Baj-Krzyworzeka et al., 2006]. In this study, TMV were labeled with PKH-26 red dye and were added to human monocytes followed by incubation. Crystal violet solution was used for quenching extracellular fluorescence coming only from membrane-attached TMV [Van Amersfoort & Van Strijp, 1994]. After 24h, strong red fluorescence was observed which was not quenched by crystal violet [Fig. 6]. This suggests that by that time most of TMV localized intracellularly. Thus TMV not only adhere to cell membrane (transfer of receptors) but are also effectively engulfed. By the use of other methods e.g. live-cell fluorescence microscopy, it was indicated that exosomes were internalized through endocytosis pathway, trapped in vesicles and transported to perinuclear region, but not to the nucleus. The inverted transport of lipophilic dye from perinuclear region to cell peripheries was revealed, possibly caused by recycling of the exosome lipids. The idea that TMV or other MV proteins are re-expresed (recycled) on cell membrane after engulfment is extremely attractive but still not proven [Muralidharan-Chari at al., 2010]. Outward transport of exosome lipids was presented by Tian et al. [Tian et al., 2010]. The authors suggested the separation of exosome lipids and proteins as lipids are recycled and proteins were trapped in lysosomes. They also suggested that exosomes internalization did not occur through the fusion [Tian et al. 2010].

Similar studies with the used of fluorescently labeled exosomes injected into the footpads of mice were performed by Hood et al. to follow nodal trafficking of melanoma exosomes [Hood et al., 2011]. The authors present (using fluorescent microscopy) a novel tumor exosome dependent model of lymphatic metastatic progression that supports the hypothesis that exosomes may be instrumental in melanoma cell dissemination [Hood et al. 2011].



Monocytes engulfed PKH-26 labelled TMV

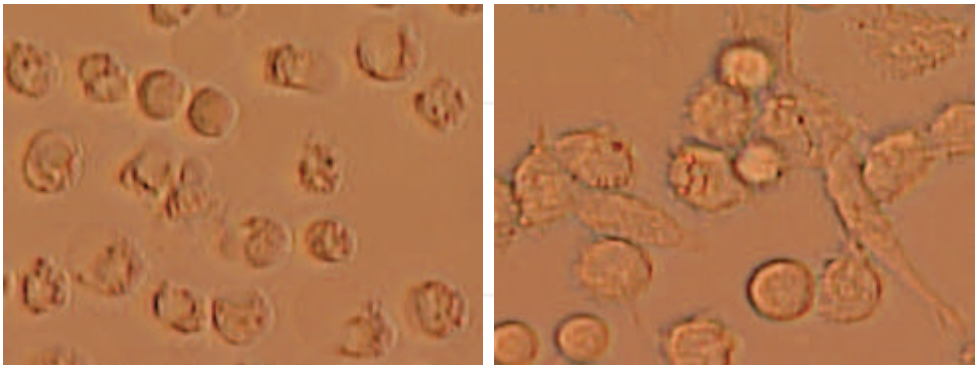
Crystal violet did not change strong red fluorescence of monocytes. Monocytes in medium (dotted line) or with PKH-26 labelled TMV (bold line) followed by crystal violet quenching.

Fig. 6. Tracking of fluorescently labeled TMV in human monocytes. Left panel presents confocal microscopy image after 24h incubation of PKH26 labeled-TMV with human blood monocytes; right panel presents the assessment of crystal violet quenching in these cells by flow cytometry [Baj-Krzyworzeka et al., 2006, modified)

5.3 TMV/exosomes interactions with the immune system cells (monocytes, lymphocytes, dendritic cells)

It is widely accepted that the immune system can control tumor growth especially at the early phases of its development [Dun, et al. 2004]. Based on our observations TMV may activate blood monocytes as judged by a significant increase in HLA –DR expression (higher MFI) and morphological changes [Fig. 7]. Monocytes are a heterogeneous population of blood cells. In particular, the different expression of CD14 and of CD16 is used to define the major subsets, the so called "classical' $CD14^{++}$ $CD16^{-}$ MO, typically representing up to 90-95% of all MO, and 'non-classical' $CD14^{+}CD16^{++}$. The $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{++}$ monocyte subpopulations interact with TMV with different results e.g. $CD14^{++}CD16^{-}$ engulfed more TMV than $CD14^{+}CD16^{++}$ cells [Fig. 8, Baj-Krzyworzeka et al., 2010]. However, at some point of tumor growth control process, immune surveillance of tumors fails, leading to the local or systemic progression of the tumor. The escape mechanisms adopted by the tumor cells lead to silencing of their immunogenic profile by activating immunosuppressive/ deviating pathways. The mechanisms by which cancer cells escape immune surveillance is still unknown, however, growing evidence suggests that TMV as well as MP released by the immune cells may play an important role in this phenomenon. It has been hypothesized that

TMV shedding may be a way for the tumor cells to dispose of "unwanted", immunogenic molecules from their surface. Indeed, apoptosis-inducing proteins and terminal components of complement have been shown to be shed *via* TMV in some tumors [Abid Hussein et al. 2007; van Doormaal, et al. 2009; Camussi et al. 1987; Sims et al. 1988]. Moreover TMV can modulate the function of tumor infiltrating lymphocytes *via* FasL expression which



(A) (B)

Fig. 7. Light microscopy image of human monocytes cultured for 24h alone (A) or in the presence of TMV (B)

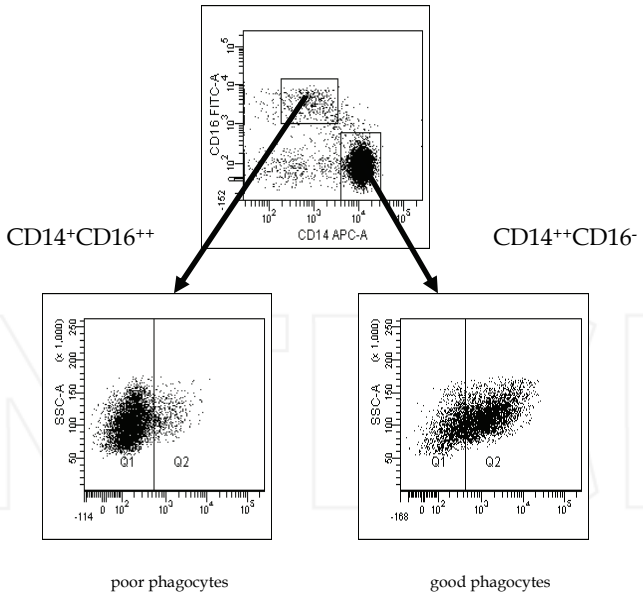


Fig. 8. Engulfment of PKH-labeled TMV by monocyte subpopulations. Flow cytometry analysis of $CD14^{++}CD16^{-}$ (right panel) and $CD14^{+}CD16^{++}$ (left panel) cells exposed to PKH26 labeled-TMV_{HPC} for 18 h followed by quenching of extracellular fluorescence with crystal violet. [Baj-Krzyworzeka et al., 2010, modified]

induce apoptosis of Fas-bearing immune cells [Whiteside, 2005, Abrahams et al., 2003, Bergmann C., et al. 2009]. Suppressive activity of TMV was also described by Szajnik et al. [Szajnik et al., 2010]. In this study, TMV induced generation of Treg and enhanced their expansion. TMV mediate the conversion of CD4+CD25neg T cells into CD4+CD25high FOXp3+ Treg as judged by flow cytometry. Tumor-derived MP derived from melanoma and colorectal carcinoma that expressed TRAIL, are responsible for apoptosis of tumor-specific T cells [Iero M. et al. 2008]. Natural killer (NK) cells upon contact with TMV lose their cytolytic potential through the downregulation of perforin expression [Liu C., et al. 2006]. Blood-derived exosomes from melanoma patients have been shown to promote the generation of myeloid-derived suppressor cells (MDSCs) from peripheral blood monocytes [Frey 2006]. MDSCs have potent immunosuppressive functions that can suppress T cell immune responses by a variety of mechanisms [Soderberg et al., 2006, Liu et al., 2010].

5.3.1 Flow cytometry detection of cytokine and chemokine secretion by monocytes stimulated with TMV

Simultaneous analysis of many parameters/factors in one sample is possible by modern bead-based immunoassays called cytometric bead array. Based on the broad range of fluorescently labeled beads coated with specific capture monoclonal antibodies, the measurement of multiple proteins from a small volume of a single sample, such as serum or culture supernatants became possible. Each bead population in the array has unique fluorescence intensity so the beads can be mixed and run together in one tube. Such systems are accessible from different manufacturers, e.g FlexSet (BD Bioscience) or xMAP (Luminex Corporation) and are compatible with different flow cytometry systems. The FlexSet beads are discriminated in FL-4 (red) and FL-5 (far red) channels, while the concentration of specified proteins, e.g. cytokines are determined by anti-cytokine PE-conjugated detection antibodies to form complexes (Fig. 9). The intensity of FL-2 (orange) fluorescence (due to anti-cytokine PE-conjugated monoclonal antibodies binding) is directly proportional to cytokine concentration in the sample which is calculated from standard curves.

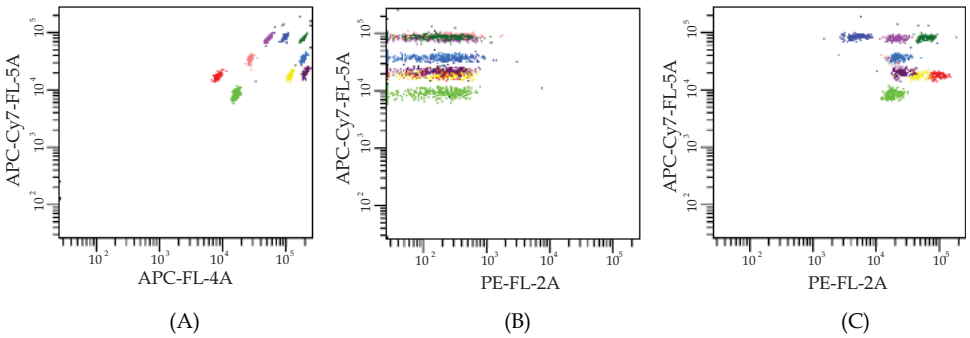


Fig. 9. Flow cytometry bead array analysis of nine different soluble factors. A – beads discrimination according to FL4 and FL5 fluorescence, B- negative control sample, C- positive sample. Analysis performed by FACSCanto flow cytometer

For our purposes we use FlexSet system followed by FACSCanto analysis of chemokine and cytokine secretion by monocytes stimulated with TMV (Fig. 10). This technique is fast and credible, but there are some limitations to this method. For instance, it is difficult to adjust the concentration of all tested parameters in one sample to be in the detection range [Baj-Krzyworzeka et al., 2011].

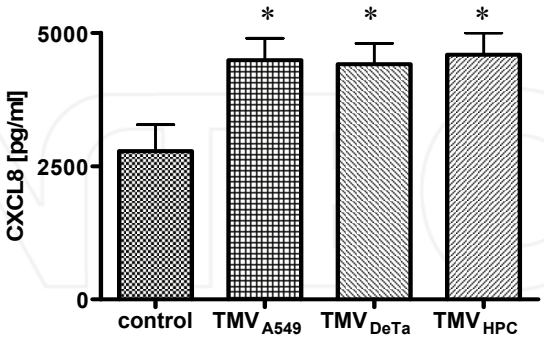


Fig. 10. The secretion of CXCL8 (IL-8) by monocytes cultured alone (control) or stimulated with TMVs after 18 h culture. The level of chemokine in the culture supernatants was determined by FlexSet method. Data (mean±SD) from four independent experiments are presented. *p<0.05 compared to the control [Baj-Krzyworzeka et al., 2011, modified]

5.3.2 Intracellular production of reactive oxygen (ROI) and nitrogen (RNI) intermediates measured by flow cytometry

The intracellular production of O_2^- and H_2O_2 may be easily and effectively measured by flow cytometry using oxidation-sensitive fluorescent probes, such as hydroethidine (HE, Sigma) and dihydrorhodamine 123 (DHR123, Sigma), respectively [Baj-Krzyworzeka et al., 2007]. Similarly to tumor cells, TMV induce ROI production by monocytes. Using flow cytometry we were able to establish that $CD14^{++}CD16^-$ cells are the major producers of ROI [Baj-Krzyworzeka et al., 2007, 2010].

Production of RNI (NO) may be also assessed by flow cytometry using intracellular staining diaminofluorescein-2 (DAF-2). A significantly higher percentage of DAF-2 positive cells was found among $CD14^+CD16^{++}$ cells in comparison to $CD14^{++}CD16^-$ cells suggesting that the former are the main producers of NO [Baj-Krzyworzeka et al., 2010].

5.3.3 Chemotactic activity of blood leukocytes induced by TMV measured by flow cytometry

TMV were described to induce chemotaxis of blood leukocytes [Baj-Krzyworzeka et al., 2011], fibroblasts and endothelial cells [Wysoczynski et al., 2009, Castellana et al., 2009]. For non-adherent cells, their chemotactic activity can be measured in Transwell 24-well plates with adequate size (e.g. 5 or 8 µm) pore filter (Costar Corning, Cambridge, MA, USA) followed by flow cytometry analysis. The cells were gated according to their FSC/SSC parameters and counted during a 20 s acquisition period at the high flow rate. Also phenotyping of migrating cells is possible. Data are expressed as the percentage of the cell

input corrected by the percentage of cells which migrated spontaneously to the medium. [Baj-Krzyworzeka et al., 2011]. Chemotactic migration of granulocytes, monocytes and lymphocytes to TMVA549 assessed by flow cytometry is presented in Fig. 11.

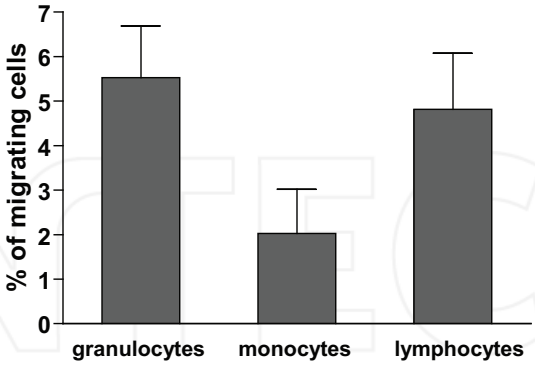


Fig. 11. Chemotaxis of human peripheral blood leukocytes to TMV_{A549} analyzed by Transwell assay followed by flow cytometry counting. Data are expressed as the percentage of the cell input number corrected by the percentage of cells, which migrated spontaneously to the medium alone. Results (mean±SD) from four independent experiments are presented. [Baj-Krzyworzeka et al., 2011 modified]

6. A new perspective in MP studies by flow cytometry

6.1 Future of instrumentation

The inability of conventional flow cytometers to measure objects smaller than 200 nm uncovers a technological gap which results in inadequate information with regards to MP data acquisition [Dragovic et al., 2011, Vasco et al., 2010]. As the MP field is becoming more attractive to the scientific community, with its wide-range applications in many types of medical fields especially, there is a growing interest of companies in the development of appropriate technology that would enable to study the whole spectrum of MP.

At the same time it must be pointed out that even with the resolution adjustments of these flow cytometers the MP fraction of 100 nm and less in size (exosomes) is still undetectable by direct measurement, which only stresses out that there is still room for improvement in this technology. An alternative way to assess MP employs the nanoparticle tracking analysis technology with the size resolution being at app. 50 nm [Dragovic et al., 2011, Vasco et al., 2010]. The principal behind it involves a finely focused laser beam that is introduced to a diluted sample through a glass prism. The laser beam then illuminates the particles in the sample and their images are transmitted through a camera mounted on a microscope onto a computer screen. The result is a short video (a compilation of frame-by-frame images), that is analyzed with appropriate computer software to first identify particles and then track their Brownian movement. The measured velocity of the particle movement is then used in calculating particle size by applying two-dimensional Stokes-Einstein equation. Additionally, this technology enables counting of particles as well as it can be equipped with modules measuring particles zeta potential and fluorescence signals when stained with appropriate antibodies conjugated with different fluorochromes.

6.2 MP as biomarkers

Monitoring of MP (PMP, TMV) level in plasma and other body fluids may be informative for effectiveness of anti-cancer treatment and for prediction of distant metastases. Also, measurement of protein composition of MP (phenotype analysis) may be useful to monitor the efficacy of anti-cancer treatment as therapeutic drugs are expulse from tumor cell *via* MP. Additionally, protein composition of MP might reflect molecular changes in tumor cells.

The whole concept behind studying MP appeared from an idea that they may represent another mode of cell-to-cell communication. The active release of MP by one cell type to another with specific surface determinant composition and internal cargo may have a profound impact on many types of normal and pathological conditions. This form of interpretation of the MP release may explain many phenomena that at present are not well-understood, i.e. angiogenesis, metastasis, M2 polarization of macrophages, etc.

Although the use of MP as biomarkers is not a gold standard, however, a number of reports shows that in certain situations they should be taken under consideration as predictors of various pathological conditions.

There is an increasing evidence that an elevated MP number is associated with certain diseases, which in itself may serve as a feasible predictor. For example, Kim et al. reported that the level of circulating platelet-derived MP is elevated in patients with gastric cancer [Kim et al., 2005]. An increase in MP number was also observed in several cardiovascular pathologies including stroke, hypertension and acute coronary syndromes [VanWijk et al., 2003]. Another group showed that an elevated MP number might be responsible in the development of atherosclerosis [Diamant et al., 2004]. Although most of the data regarding elevated MP numbers comes from platelet-derived MP, which constitute about 80% of the total blood MP population, however, MP may be also generated by other cell. An elevated MP number derived from endothelial cells was observed in arterial stiffness in hemodialysed patients [Amabile et al., 2005]. Fibroblasts were also shown to shed abundant numbers of MP in rheumatoid arthritis [Distlar et al., 2005]. Monitoring of MP levels is not restricted to blood only. Other bodily fluids may also serve as a source of information on elevated MP numbers. For instance, recent discovery of elevated MP numbers present in urine in patients developing acute kidney injury may also point to the usefulness of the MP level monitoring [Zhou et al., 2006]. Also, malignant effusions such as ascites fluids were also shown to have elevated MP numbers [Andre et al., 2002].

Besides the assessment of elevated MP numbers what seems to be even more promising with respect to biomarker monitoring is to try to establish their phenotypic/internal cargo composition and, hopefully, correlate it with appropriate pathological conditions. Tissue factor (TF) (responsible for coagulation) is overexpressed in many types of cancers, i.e. bladder, brain, colon, gastric [Patry et al., 2008, Brat & Van Meir et al., 2004, Hron et al., 2007, Yamashita et al., 2007]. TF^+ MP were observed in blood of patients with colorectal cancer which were trapped and then fused with the membrane of activated platelets thus propagating and even initiating coagulation which in turn favored tumor cell invasiveness and metastasis [Hron et al., 2007]. Also, MP bearing CD147/extracellular metalloproteinase (MMP) inducer derived from ovarian cancer cells stimulated proangiogenic activities of human umbilical vein endothelial cells (HUVECs) [Millimaggi et al., 2007]. In inflamed and

atherosclerotic endothelium, it was shown that platelet-derived MP transported and deposited substantial amounts of RANTES (CCL5) promoting monocyte recruitment [Mause et al., 2005]. Dendritic cell-derived MP (Dex) were shown to deliver peptide-loaded MHC class I/II molecules to naïve DC, which in turn were able to fully stimulate cognate T cells [Krogsgaard & Davis et al., 2005]. Circulating MP have been also reported to be responsible for an inflammatory response in sepsis. It has been shown that they may provoke endothelium inflammation by lysophosphatidic acid and thus stimulating chemotactic migration of platelets/leukocytes to the endothelium. This in turn may lead to the production of monocyte cytokines such as IL-1β, IL-8 and tumor necrosis factor-α resulting in further inflammation enhancement [Mortaza et al., 2009, Lynch & Ludlam, 2007, Gambim et al., 2007]. MV monitoring may be also beneficial in the case of venous thrombosis in cancer patients. Tumor cells (different types of tumors) often are characterized by high procoagulant potential mostly due to the overexpression of surface TF [Ran & Thorpe, 2002, Yu et al., 2005, Dvorak & Rickles, 2006]. Release of TF^+ MV from the tumor cells into circulation may be the cause of coagulation system activation by generating thrombin which in turn results in the formation and subsequent deposition of fibrin in blood vessels thus creating a favorable niche for metastasis [Rak et al., 2006, Hron et al., 2007, Tesselaar et al., 2007]

Altogether, looking at MP as biomarkers may be useful in a proper assessment of a number of diseases and serve as a feasible explanation of certain biological processes.

7. Conclusion

Flow cytometry should be considered as a method of choice for detection and analysis of MP in biological fluids. Polychromatic flow cytometry analysis, especially, is recommended for establishing a cellular source and antigen composition of analyzed MP. However, for exosomes characterization, standard flow cytometers, due to their detection limits, are not suitable. In this case, some other approaches, such as adsorption of exosomes to anti-tetraspanin coated latex beads, may be introduced thus overcoming limits of currently available instrumentation. In addition to MP analysis, flow cytometry allows enumeration of their numbers in biological samples. This possibility seems to be of importance as absolute numbers of MP may have a clinical relevance in monitoring life threatening diseases such as cancer, sepsis and thrombosis.

8. Acknowledgment

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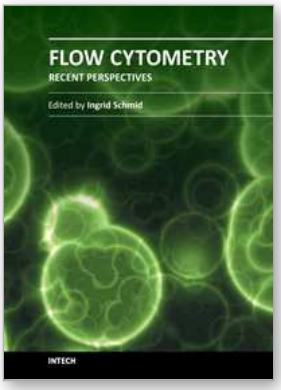

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"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821