

IN VITRO STUDY OF PRO-INFLAMMATORY AND ANTI-TUMOUR PROPERTIES OF MICROVESICLES FROM BACTERIAL CELL WALL OF *PANTOEA AGGLOMERANS*

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Abstract: In the environment, Gram-negative bacteria are capable of producing large amounts of endotoxin-containing microvesicles – spherical structures measuring 30–50 nm in diameter, emerging by fragmentation of the outer membrane of the bacterial cell wall. Microvesicles are suspected of inducing inflammatory lung diseases, but possibly also of stimulating anti-tumour defence mechanisms. The present study was aimed at assessing the pro-inflammatory and anti-tumour properties of microvesicles *in vitro*. Peripheral blood mononuclear cells of 5 healthy volunteers were cultured for 6 h, 24 h, 3 days, and 5 days with microvesicles (MV) of *Pantoea agglomerans* at concentrations ranging from 0.48–1500 µg/ml. The following outcomes were measured: secretion of IFN-γ and TNF-α (by ELISA and ELISpot), intensity of cell proliferation (LPT), expression of surface markers CD8, CD14, CD16, CD25, CD69, CD80, CD83, HLA-DR, and apoptosis markers (by flow cytometry). After 24 hours, a clear dose-dependent response to microvesicles was seen for IFN-γ production, starting already at the lowest concentration of 0.48 µg/ml (p=0.04). A 2-fold increase in TNF-α production was seen after 3 days at the concentration of 1,500 µg/ml (p=0.05). A clear and significant dose-dependent increase in cell proliferation in response to MV was detectable after 5 days (p=0.001). A decrease in the percentage of CD14(+)CD83(+) monocytes was observed after 1 day of culture. We conclude that IFN-γ and TNF-α are triggered at different concentrations of microvesicles: at lower concentrations only IFN-γ is upregulated, whereas at higher concentrations both IFN-γ and TNF-α are secreted.

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INTRODUCTION

Exposure to organic dust is widely accepted as a major cause for a range of lung disorders, including organic dust toxic syndrome (ODTS, syn. toxic pneumonitis), chronic obstructive pulmonary disease (COPD), increased airway responsiveness, asthma, and granulomatous pneumonitis (syn. allergic alveolitis, hypersensitivity pneumonitis) [8, 31, 35, 39, 42]. A connection has also been described between organic dust exposure and inflammatory skin disease

[5, 22, 51, 52, 53, 56, 57, 58, 59]. Epidemiological data suggest, however, also a possible positive effect of organic dust exposure on human health: workers exposed to organic dust seem at lower risk for lung cancer [29, 30]. Also, exposure to organic dust in early life is regarded to reduce atopy and prevent respiratory allergies in children [40, 42]. Among candidate components responsible for both adverse and beneficial biological effects of exposure to organic dust are Gram-negative bacteria and their toxins, of which lipopolysaccharides (LPS, syn. endotoxins) attract

the most attention of researchers [10, 21, 27, 31, 35, 36, 40, 42, 43, 44, 64]. Disruption of Gram-negative bacteria produces large amounts of spherical, endotoxin-containing structures measuring 30–50 nm in diameter. These structures, referred to as “microvesicles” (MV) or “microglobules”, emerge by fragmentation of the outer membrane of the bacterial cell wall. They occur abundantly in wood dust, grain dust and other organic dusts [13, 16, 17]. Animal studies have demonstrated that repeated inhalation exposures to endotoxin-containing microvesicles (MV) lead to substantial increase of circulating interferon (IFN) and tumour necrosis factor alpha (TNF- α), significantly higher than in animals exposed to respective doses of pure endotoxin [15, 45].

The aim of the present work was to study *in vitro* the pro-inflammatory and anti-tumour properties of endotoxin-containing microvesicles (MV) on human leukocytes. The present study focused on the following possible effects of microvesicles: secretion of interferon gamma (IFN- γ) and tumour necrosis factor alpha (TNF- α), proliferation, and expression of cell surface markers on human peripheral blood mononuclear cells (PBMC).

MATERIALS AND METHODS

Production of microvesicles. Microvesicles were produced as described earlier [15, 16, 45]. They were separated from bacterial mass in sucrose density gradients, prepared with the Hoefer SG Gradient Maker (Hoefer Scientific Instruments, San Francisco, CA, USA). *Pantoea agglomerans* (synonyms: *Enterobacter agglomerans*, *Erwinia herbicola*), strain Z-11, originally isolated from settled grain dust, was grown on nutrient agar in Roux bottles, for 72 h at 35°C. Bacterial cell mass was harvested from each bottle with 12 ml phosphate buffered saline, pH 7.4, containing 0.001% gelatine. The washes from 10 bottles were pooled and centrifuged. The resulting bacterial pellet was then resuspended in 5 ml buffer, and thereafter each 1 ml was placed on 20 ml sucrose density gradient (ranging from 5–30%) and centrifuged for 15 min at $3,500 \times g$ in a 25 ml tube. After this procedure, a pellet of whole cells was visible and a broad turbid layer in the lower third of the gradient, consisting mostly of particles smaller than whole cells. This layer was carefully decanted, pelleted by centrifugation, resuspended in 0.5 ml buffer, placed in a tube with sucrose gradient of higher density (6 ml, range from 10–45%), and centrifuged again at $3,500 \times g$ for 30 min, which resulted in the formation of a more defined band about 5–8 mm thick, in the lower quarter of the tube (Fig. 1). This fraction was decanted, centrifuged, checked by electron microscopy (Figs. 2–3), and finally lyophilized as microvesicles (MV) for further studies. The endotoxin activity of MV was measured using the Limulus Amebocyte Lysate (LAL) test, as described elsewhere [46], and the endotoxin content finally calculated as 31.25 mg pure endotoxin per 1 g MV (3.125%).

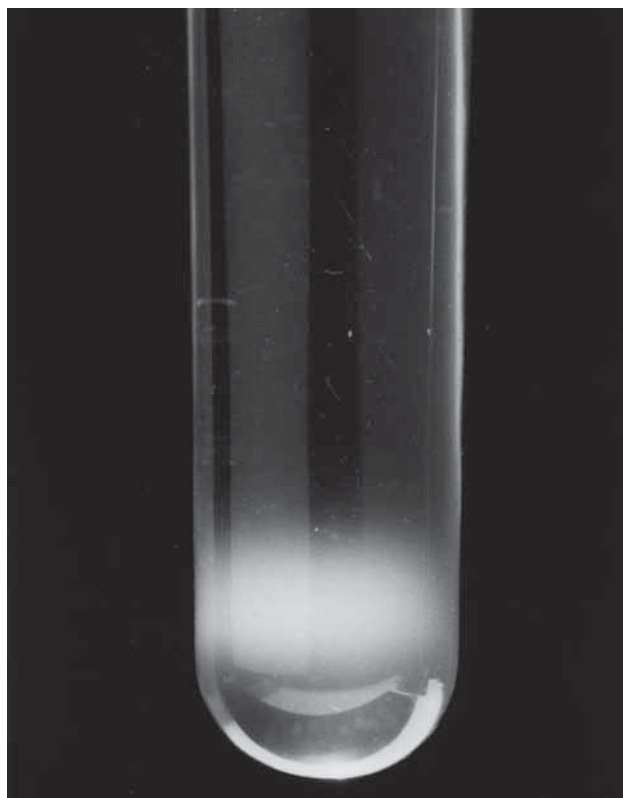


Figure 1. Turbid layer above the bottom of the tube consists of microvesicular particles, separated in sucrose density gradients.

Cell cultures. Peripheral blood mononuclear cells (PBMC) used for the experiments were obtained from 5 healthy volunteers (2 men and 3 women, aged 38–51 years) with no history of exposure to organic dusts. PBMC were separated from the participants' blood samples through density gradient centrifugation in Ficoll-Paque Plus (Amersham, Uppsala, Sweden). Immediately after separation and washing, the cells were counted and tested for viability by trypan blue exclusion ($\geq 97\%$ viable cells were found in each cell batch). The cells were cultured at 37°C, 95% RH and 5% CO₂ in IMDM medium (Cambrex, Verviers, Belgium) supplemented with penicillin 50 U/ml and streptomycin 50 $\mu\text{g}/\text{ml}$ (Lonza, Verviers, Belgium) and 1,4-dithiothreitol 7.8 $\mu\text{g}/\text{ml}$ (Sigma-Aldrich Chemie, Steinheim, Germany) [55]. The final cell density was 5×10^3 cells/well (25,000 cells/ml) for ELISpot assay, and 10^5 cells/well (500,000 cells/ml) for ELISA and LPT assays.

Enzyme-Linked Immunosorbent Assay (ELISA). PBMC for ELISA were cultured at 10^5 cells/well in triplicate for 6 h, 24 h, 3 days, and 5 days, without addition of MV and with each concentration of the 5-fold dilution series of MV: 0.48, 2.4, 12, 60, 300, and 1,500 $\mu\text{g}/\text{ml}$. After an appropriate time, supernatants were collected and kept at -20°C until cytokine determination was performed with ELISA. IFN- γ and TNF- α were measured with PeliKine™-compact human IFN- γ and TNF- α ELISA kits, respectively

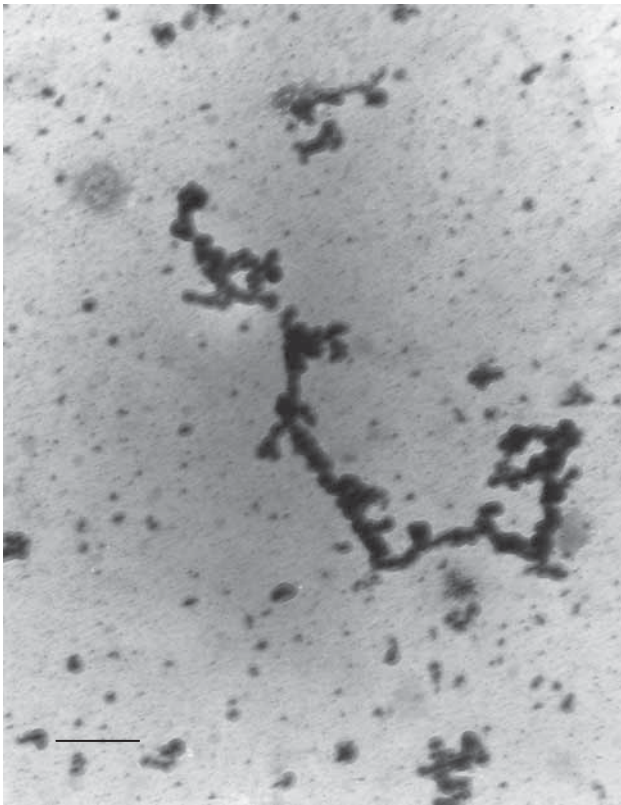


Figure 2. Ultrastructure of microvesicular particles separated from the bacterial mass of *Pantoea agglomerans*. Note the linear aggregation of the particles. Bar, 0.2 μm .

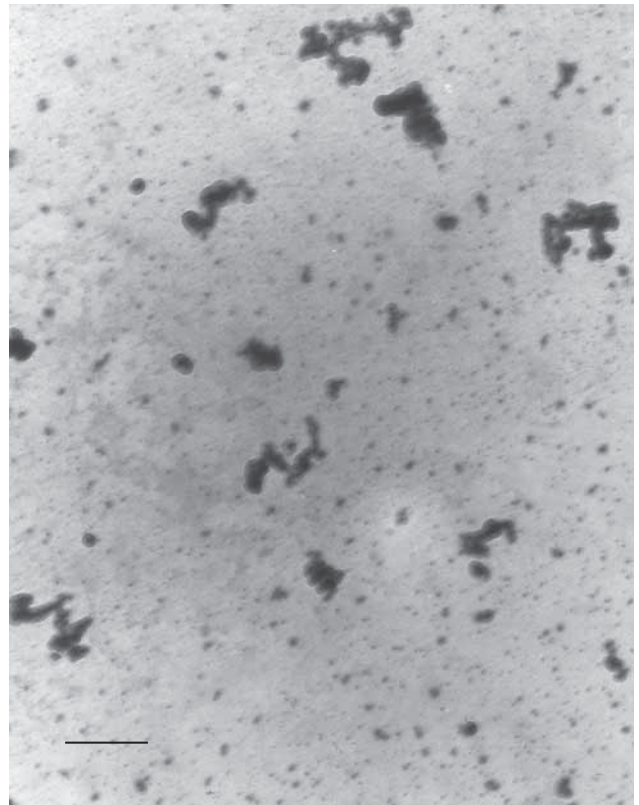


Figure 3. Ultrastructure of microvesicular particles separated from the bacterial mass of *Pantoea agglomerans*. Note the small aggregations of the particles. Bar, 0.2 μm .

and PeliKine™-Tool supplementary reagents set (Sanquin, Amsterdam, The Netherlands), according to the manufacturer's recommendations. According to manufacturer's information, the detection levels for both cytokines were 1 pg/ml. The absorbance was measured at 450 nm wavelength with Microplate Autoreader ELX808, and the final cytokine concentrations were calculated automatically by the KC Junior Software (Bio-Tek, Winooski, VT, USA).

Enzyme-Linked Immunospot Assay (ELISpot). Coating antibodies and detection antibodies for IFN- γ and TNF- α , and streptavidin-alkaline phosphatase (s-ALP) conjugate were supplied with ELISpot kits (Mabtech, Näckä, Sweden). The cells were cultured at 5×10^3 cells/well in 96-well PVDF microfilter plates (Millipore, Molsheim, France), each preincubated with coating antibodies for IFN- γ or TNF- α , following the manufacturers' recommendations. For each donor, PBMC were cultured in triplicate for 6 h, 24 h, 3 days, and 5 days, without addition of MV and with each concentration of a 5-fold dilution series of MV: 0.48, 2.4, 12, 60, 300, and 1,500 $\mu\text{g}/\text{ml}$. At the end of the culture period, the cells were disposed from the plates. Cytokines bound to the membrane during the culture were visualized using respective biotinylated detection antibodies, s-ALP conjugate, and finally the BCIP/NBT colour substrate (Mabtech, Näckä, Sweden). Spots on the membrane, each denoting one cell secreting cytokine of interest, were counted automatically

using the A.EL.VIS Eli.Scan Elispot scanner with Eli.Analyse software (A.EL.VIS, Hannover, Germany). The ELISpot technique is capable of detecting 1 cell secreting cytokine of interest out of 1 million cultured cells [48].

Lymphocyte proliferation test (LPT). PBMC were cultured at 10^5 cells/well in triplicate for 6 h, 24 h, 3 days, and 5 days, without addition of MV and with each concentration of the 5-fold dilution series of MV: 0.48, 2.4, 12, 60, 300, and 1500 $\mu\text{g}/\text{ml}$. The radiolabel for proliferation, ^3H -thymidine (Amersham, Little Chalfont, UK) was added to the cultures at the starting point with the final radioactivity 0.625 $\mu\text{Ci}/\text{well}$. After an appropriate time, the cell cultures were frozen and kept at -20°C until measurement. The amount of incorporated radioactivity was measured using microplate scintillation counter TopCount NXT with scintillation fluid Microscint O (Packard Biosciences, Downers Grove, IL, USA).

Flow cytometry analysis (FACS). Due to high workload and cost of the analyses, the number of donors tested in this part of the study was limited to 3, and the tested range of MV concentration was 0.48–300 $\mu\text{g}/\text{ml}$ with a 25-fold step. Donors' PBMC were cultured at 10^6 cells/ml (5 ml per culture) for 1, 3, and 5 days without addition of MV and with each 0.48, 12, and 300 $\mu\text{g}/\text{ml}$ microvesicles. After the appropriate incubation periods, cell samples

(each 10^5 cells) were stained for flow fluorocytometric analyses with following antibodies: anti-CD8-Cy5 (Sanquin, Amsterdam, The Netherlands), anti-CD14-FITC (BD Pharmingen, San Diego, CA, USA), anti-CD16-PE (Sanquin), anti-CD25-FITC (BD Pharmingen), anti-CD69-Cy5 (BD Pharmingen), anti-CD80-PE (BD Pharmingen), anti-CD83-PE (Beckman Coulter, Fullerton, CA, USA), anti-HLA-DR-FITC (BD Pharmingen). In order to check for non-specific binding, the cell samples were stained with respective isotype antibodies. For detection of apoptotic cells, the samples were stained with Annexin-V-FITC (Bender MedSystems, Vienna, Austria), and propidium iodide (PI, Sigma-Aldrich, Saint Louis, MO, USA). The staining procedure was carried out following recommendations of respective manufacturers. The flow cytometric measurements were carried out using FACSCalibur 3 CA (Becton-Dickinson, Mountain View, CA, USA) with Cell Quest software for data acquisition.

Data analysis. In order to detect differences in multiple related samples (series of MV dilutions), the Friedman test was carried out with raw data [23]. Statistical package SPSS+ for Windows (SPSS Inc, Chicago, IL, USA) was used for the above analyses. As there were big inter-individual differences in IFN- γ and TNF- α secretion between donors (up to 27-fold), the ELISpot, ELISA, and LPT results were presented on the graphs as indexes, i.e. results for cultures with various MV concentrations were each divided by values measured in the negative controls (culture without addition of MV). Regarding flow cytometry analyses, due to previously-mentioned reduction in the number of donors and measuring points on the dose-response curve, a statistical estimation of significance was not feasible. Therefore, only dose-dependent phenomenon consistently seen in all 3 donors was arbitrarily considered as relevant and presented in this article.

RESULTS

In ELISA tests, the measured concentrations were within the range of 1–2495 pg/ml for IFN- γ , and 61–5087 pg/ml for TNF- α with up to 27-fold differences in baseline values observed between donors. In order to make comparisons possible, trends in Figure 4 are demonstrated as means of individual values indexed against the mean baseline (negative control, i.e. spontaneous cytokine secretion by unstimulated cells). A clear dose-dependent response to MV in IFN- γ production was seen after 24 hours, with a more than 2-fold increase observed already at the lowest MV concentration tested: 0.48 $\mu\text{g/ml}$ ($p=0.042$). As for TNF- α , a significant ($p=0.05$) increase in the cytokine production was seen after 3 days, with a 2-fold increase only at the highest MV concentrations of 1,500 $\mu\text{g/ml}$. The ELISpot tests results have confirmed the ELISA results (Fig. 5). A clear and significant dose-dependent increase in cell proliferation in response to MV was detectable after 5 days ($p=0.001$).

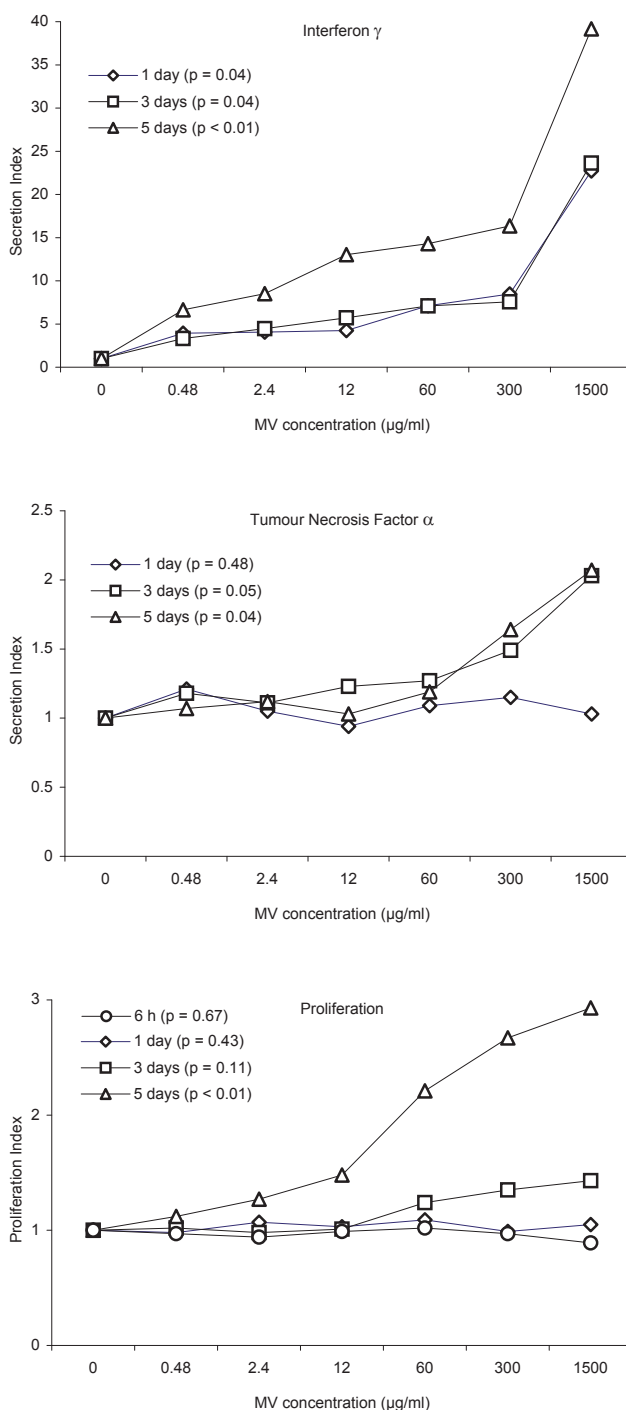


Figure 4. Secretion of IFN- γ and TNF- α by peripheral blood mononuclear cells (PBMC) and cell proliferation in response to microvesicles. Results are indexed to baseline (negative control). Note the distinct scale ranges on the graphs.

Regarding expression of cell surface markers, samples from only 3 donors were tested at this stage due to high workload and cost of analyses with a broad array of the markers. Therefore, an estimation of statistical significance was not feasible and only dose-dependent phenomena consistently seen in all 3 donors were considered relevant.






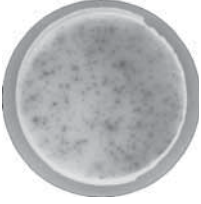

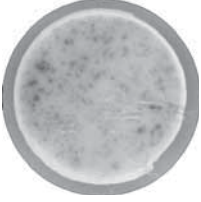

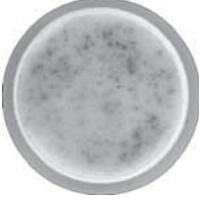

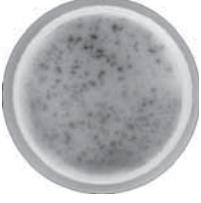

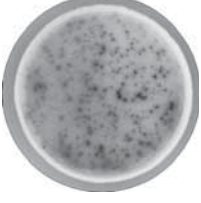

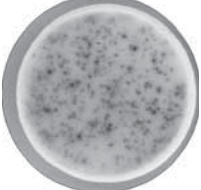
MV concentration	IFN- γ after 1 day	Spot count	TNF- α after 3 days	Spot count
0 (negative control)		66 spots/well (0.13% IFN- γ secreting cells)		87 spots/well (0.17% TNF- α secreting cells)
0.48 $\mu\text{g/ml}$		91 spots/well (0.18% IFN- γ secreting cells)		80 spots/well (0.16% TNF- α secreting cells)
2.4 $\mu\text{g/ml}$		104 spots/well (0.21% IFN- γ secreting cells)		89 spots/well (0.18% TNF- α secreting cells)
12 $\mu\text{g/ml}$		120 spots/well (0.24% IFN- γ secreting cells)		87 spots/well (0.17% TNF- α secreting cells)
60 $\mu\text{g/ml}$		133 spots/well (0.27% IFN- γ secreting cells)		91 spots/well (0.18% TNF- α secreting cells)
300 $\mu\text{g/ml}$		156 spots/well (0.31% IFN- γ secreting cells)		184 spots/well (0.37% TNF- α secreting cells)
1500 $\mu\text{g/ml}$		196 spots/well (0.39% IFN- γ secreting cells)		243 spots/well (0.49% TNF- α secreting cells)
PHA (positive control)		256 spots/well (0.51% IFN- γ secreting cells)		230 spots/well (0.46% TNF- α secreting cells)

Figure 5. Representative results for IFN- γ ELISpot after 1 day of incubation and for TNF- α ELISpot after 3 days of incubation in PBMC cultures. These were the timepoints, at which observed differences were found significant for the first time (Friedman test: $p = 0.03$, and $p = 0.04$, respectively).

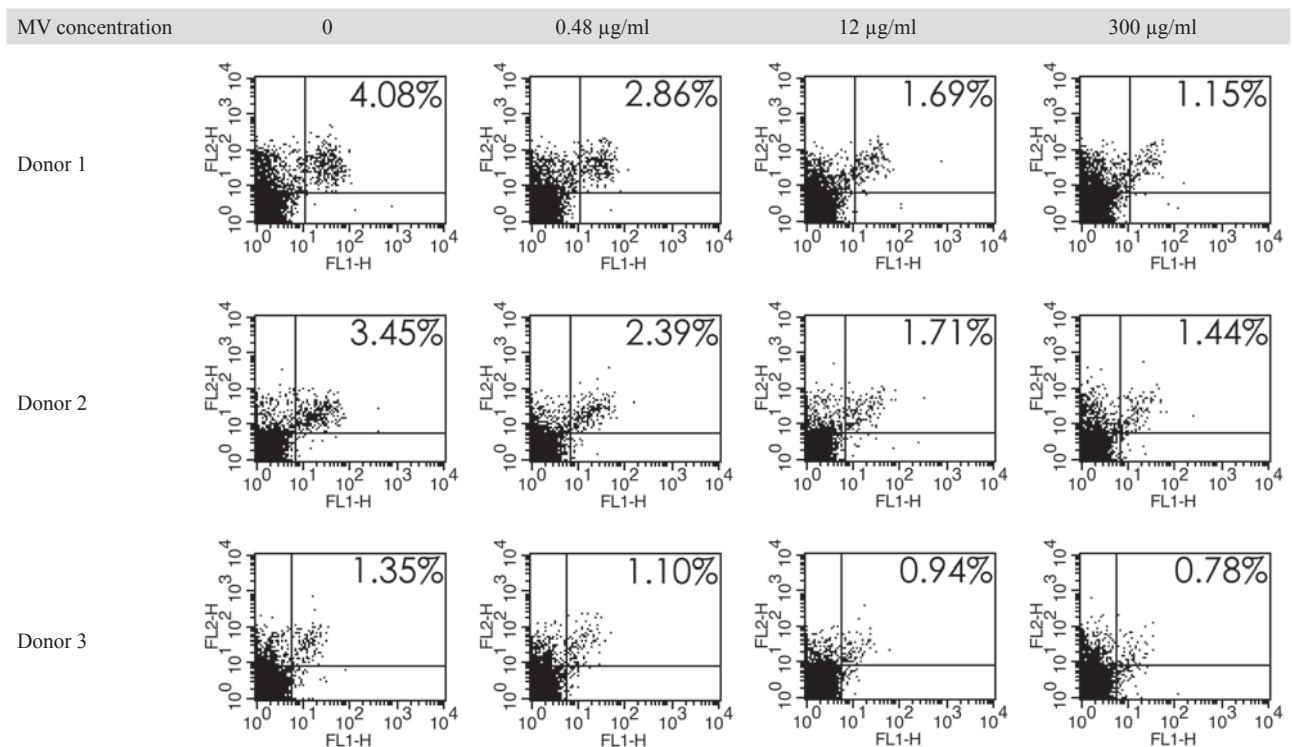


Figure 6. Changes in percentages of CD14(+)CD83(+) cells in response to 1-day culture with microvesicles. The analysed cells were gated for monocytes based on forward and side scatter. Percentages of CD14(+)CD83(+) within the gated fraction are displayed in the right upper corner of each graph.

Such a phenomenon was the decrease in percentage of CD14(+)CD83(+) cells observed after 24 h of incubation in the presence of MV. The fraction of CD14(+)CD83(+) within the monocyte fraction (defined on the basis of forward and sideward scatter of the laser beam) ranged from 0.78–4.08% with consistent dose-response relationship (decrease of the percentage at increasing MV concentrations) in all 3 donors (Fig. 6). The percentages of CD14(+)CD83(+) cells were within the range 1.08–2.67% after 3 days of culture, and 2.45–9.77% after 5 days; however, no consistent dose-response effects were seen at those time points.

DISCUSSION

In a series of experiments, we have demonstrated the dose-dependent influence of Gram-negative bacterial microvesicles (MV) on cultures of human peripheral blood mononuclear cells (PBMC). IFN- γ production was increased relatively early (after 24 hrs), and at lowest MV doses tested, whereas TNF- α became upregulated after 3 days, and only by MV doses 125-times higher. Therefore, it seems that depending on the intensity and duration of exposure, microvesicles may upregulate IFN- γ alone, or IFN- γ and TNF- α together. IFN- γ and TNF- α are potent inflammatory cytokines that act synergistically in a range of immunological processes. With respect to this study, perhaps most interesting seems their synergistic anti-tumour effect against lung cancer cells [4, 41, 61]. This might suggest that the protective effect of organic dust exposure found

in some epidemiological observations may depend on the dose of inhaled MV.

IFN- γ belongs to the family of interferons, characterised by their ability to protect cells from viral infections. Interferons are divided into 2 classes: Type I IFN includes IFN- α and IFN- β , which are the classical interferons secreted in response to viral infections. The only member of type II IFN subclass is IFN- γ (also referred to as “type II interferon” or “immune IFN”), which is neither genetically nor structurally related to the type I. IFN- γ induces most of the biologic effects typical to other interferons; however, its specific antiviral activity is lower, and immunomodulatory activity higher [18]. Moreover, IFN- γ stimulates MHC class II antigens, which is in contrast to type I interferons [1]. Cells capable of producing large amounts of IFN- γ include natural killer (NK) cells, CD4+ T helper-1 (Th1) cells, and CD8+ T cytotoxic cells [6, 18]. Less potent sources of IFN- γ are also $\gamma\delta$ T cells, NKT cells, macrophages, dendritic cells, naive CD4+ T cells, and B cells [19, 62]. According to Galin *et al.* [20], the key immunoregulatory roles of IFN- γ are: (1) improved antigen presentation; (2) enhanced killing of intracellular pathogens, which induces the synthesis of enzymes in phagocytes that are involved in the generation of reactive oxidants (eg, superoxide, hydrogen peroxide, and nitric oxide) that are crucial for the killing of intracellular and some extracellular infectious agents; (3) increased capacity for microbial killing; and (4) enhanced recruitment of leukocyte-enhanced macrophage activity, and increased intracellular concentration of antimicrobials.

TNF is a pleiotropic pro-inflammatory cytokine that plays an important role in the induction of other cytokines, cell proliferation, differentiation, necrosis, and apoptosis. TNF is either membrane-bound or secreted. Main sources are activated macrophages, lymphocytes, natural killer cells, and epithelial cells. Three classes of TNFs have been identified: TNF- α , lymphotoxin- α (LT- α), and LT- β , all of which are bioactive as trimers [37]. The biological functions of TNF- α are too many to be discussed in this article. Most important for the present study is the generally-accepted pivotal role of TNF- α in anti-tumour immunity [63]. This picture, however, is not fully clear: There are also hints that in certain situations TNF- α may paradoxically contribute to cancer development [2].

Human CD83 is a 45-kDa glycoprotein belonging to the immunoglobulin superfamily, generally accepted as surface marker for activated dendritic cells (DC) [3]. In the present study, the decrease in percentage of DC (CD14+CD83+) within the monocyte fraction after 1 day of culturing with MV may seem paradoxical. However, it can be explained with the fact that a certain fraction of LPS-stimulated CD14(+) monocytes undergoes activation into M ϕ macrophages (CD14+CD70+CD83-HLA-DR-), spontaneously producing large amounts of TNF- α and matrix metalloproteinase 9 (MMP-9) [26]. MMP-9 is collagenase, a neutral proteinase involved in the breakdown and remodelling of extracellular matrix (ECM) [9]. MMPs also cleave a variety of non-ECM proteins, including cytokines, chemokines, and growth factors, activating or inactivating these. Upregulation of matrix metalloproteinases is observed in a variety of destructive processes, including cardiovascular, inflammatory, autoimmune, and neoplastic diseases [25].

Environmental Gram-negative bacteria and their products are abundantly present in various types of occupational bioaerosols: grain dust, flax dust, wood dust, herb dust, waste and sewage aerosols [11, 12, 17]. Gram-negative bacteria and their products can also be encountered in non-occupational environments, e.g. they are present on airborne pollen [47, 54, 60]. Bacterial endotoxin typically occurs in the environment in the form of microvesicles, which are composed of lipopolysaccharides, proteins and phospholipids [7, 17]. The results of the present work show that the isolated fraction of endotoxin-containing microvesicles exerts a significant effect on the cytokine production by human cells *in vitro*. These data confirm the results of the earlier studies by Skórska *et al.* [45] and Dutkiewicz *et al.* [15] who demonstrated in animal experiments strong stimulation effects of endotoxin-containing microvesicles of the *Rahnella* spp. and *Pantoea agglomerans* on cytokine production. All these results corroborate those obtained by Rylander [38] and Milanowski [32, 33] who reported that cell-bound endotoxin shows stronger biological activity than purified LPS.

It appears that the biological activity of endotoxin also depends on the species of bacteria, and that potency of the *Pantoea agglomerans* endotoxin is greater compared to

other Gram-negative bacteria [10, 14, 24]. This species exhibits also strong allergenic properties and could be a cause of work-related respiratory and skin disorders in people exposed to organic dusts [11, 28, 34, 49, 59]. The fact that the dust-borne endotoxin of *Pantoea agglomerans* occurs in the form of fine, submicroscopic microvesicles [16, 17] easily penetrating into alveoli, increases the potential risk of disorders after exposure to these bacteria. On the other side, however, this fact might increase non-specific immunity against neoplastic diseases and suppress atopic predisposition in exposed people. The present work, demonstrating the immunomodulatory effects of *Pantoea agglomerans* endotoxin-containing microvesicles on human cells proves a possibility of both adverse and beneficiary effects in people inhaling these structures.

CONCLUSIONS

Secretion of IFN- γ and TNF- α by peripheral blood mononuclear cells (PBMC) is triggered at different concentrations of microvesicles. At lower concentrations, only IFN- γ is upregulated, whereas at higher concentrations both IFN- γ and TNF- α are secreted. Increase of IFN- γ begins between 6 and 24 hours of stimulation, whereas increased production of TNF- α begins between 1 and 3 days. These data suggest that biological effects of organic dust may be dependent of the duration of exposure and the inhaled dose, which might offer some hint for future epidemiological studies.

Acknowledgements

The ELISpot Scanner and ELISA Reader used in this study were donated by the Śpiewak Family to commemorate the late Karolina Śpiewak (1904–1989), a devoted Polish mother, whose heroic and extraordinary achievement was to ensure survival for 9 out of her 10 children from the Soviet deportation to Siberian Gulags during World War II. Initial results of this study were presented at 2 scientific conferences in 2006 [50, 51].

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