# Microparticles released by Ectocytosis from Human Neutrophils:

# Characterisation, Properties and Functions

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Olivier Gasser

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Auf Antrag von:

Professor Jürg A. Schifferli

Professor Martin Spiess

Professor Christoph Moroni

Basel, den 4 mai 2004

Professor Marcel Tanner, Dekan

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## General Summary

The field of microparticles (MPs) has gained growing interest over the last decade. Numbers of papers have come out recently describing molecular or functional characteristics of MPs derived from various cells, suggesting *in vivo* roles for MPs other than being inert side-products of cellular activation.

The properties and characteristics of MPs released from the surface of activated human polymorphonuclear neutrophils (PMN), called ectosomes, will be discussed in Part I. The functional implications of these characteristics with regards to cellular interaction of PMN-ectosomes, in particular with macrophages, and their circulation in blood will be addressed in Part II and III, respectively.

As presented in Part I, many neutrophil-derived membrane proteins were translocated to the surface of ectosomes. There was no positive or negative selection with regards to transmembrane type versus glycophosphatidylinositol-linked type of proteins. Indeed, both types had representatives present and absent on the surface of ectosomes. In addition, ectosomes exposed several active enzymes on their surface, such as proteinase-3, myeloperoxidase, elastase and matrix metalloproteinase-9. The fact that ectosomes are unable to maintain the asymmetry of their membrane bilayer was illustrated by the presence of phosphatidylserine on their outer membrane leaflet. Ectosomes were also found to bind the first component of the classical pathway of complement, C1q; an additional finding that has been looked at more in detail in Part III.

Binding assays revealed affinity of ectosomes to endothelial cells as well as macrophages. Further analyses of the interaction between PMN ectosomes and human monocyte derived macrophages (HMDM) presented in Part II provided data suggesting that ectosomes do not

only bind, but are subsequently phagocytosed by HMDM. The sole binding of ectosomes, however, was sufficient to induce an anti-inflammatory reprogramming of HMDM. In particular, ectosomes dose-dependently counteracted the pro-inflammatory response of HMDM to stimuli such as zymosan and LPS. This effect comprised an early increase in the release of the anti-inflammatory cytokine TGFβ1 and subsequent downregulation of IL-8, IL-10 and TNFα secretion. Data obtained using neutralising anti-TGFβ antibodies suggested that both phenomena might be causally linked, at least partially.

As alluded to above, ectosomes bind C1q. As presented in Part III, ectosomes were also found to activate and fix complement. C3- and C4-fragments were detected on the surface of ectosomes after incubation with normal human serum. Using C1q- and C2-deficient serum, the ectosome-induced activation of complement could be mainly attributed to the classical pathway. Finally, the opsonisation of ectosomes by complement induced their immune adherence to erythrocytes. These data suggest that blood-borne ectosomes might be sequestered on erythrocytes, a mechanism that might drive their clearance from circulation, similarly to immune complexes.

**General Introduction**

Vesiculation is a ubiquitous cellular phenomenon occurring either intra-cellularly or at the cell membrane. As one of numerous forms of vesiculation, ectocytosis is defined as the formation and release of right-side out oriented vesicles, ectosomes, directly from the cell surface. Ectocytosis is inducible in various eukaryotic cell types, including polymorphonuclear neutrophils (PMN), the cell that was focused on here. Stein and colleagues initially stimulated PMN with sublytic amounts of complement; in response the cells released ectosomes that rid the cells from pore-forming membrane attack complexes[1]. Alongside complement proteins, which triggered ectocytosis, a number of cell-derived proteins and lipids were selectively sorted into ectosomes.

Ectocytosis is distinct from another vesiculation process called exocytosis. Exosomes and ectosomes, differ in their genesis in that exosomes are preformed and stored vesicles released by fusion of so called multivesicular bodies (MVBs) with the cell membrane. Exo- as well as ecto-somes can be released from a single cell, as for instance the activated thrombocyte[2].

The molecular mechanisms of ectocytosis are still largely unknown, as are the various functions ectosomes might have for each cell-type they derived from. However, data have accumulated recently suggesting that ectosomes act as vesicular mediators that adopt cell-specific functions, most of which being of pro-coagulant or pro–inflammatory nature. Described functions range from inter-cellular transfer of CCR5 and tissue factor from mononuclear cells and platelets, respectively, rapid IL-1β shedding from monocytes, endothelial cell and monocyte activation induced by monocytes and platelets, to extracellular matrix remodeling by ectosomes released by fibroblasts and chondrocytes[3-9]. The most described features of ectosomes, mainly attributed to ectosomes released by platelets and monocytes, are their ability to promote coagulation and activate endothelial cells[7,8].

Microparticles (MPs), likely to correspond mostly to ectosomes, were shown to directly support thrombus formation in a tissue factor dependent manner[10,11].

To find, possibly causative, links between MPs, including ectosomes, and homeostasis, MPs were traced in serum from patients suffering from various diseases such as sepsis[12], severe trauma[13], paroxysmal nocturnal hemoglobinuria[14], diabetes[15,16], acute coronary syndromes[17] and lupus[18]. Although MPs could not be clearly implicated in the pathophysiology of these diseases, significant quantitative as well as qualitative changes in MP-counts were observed in health versus disease. PMN-ectosome-counts were in particular found elevated in situations where PMN are systemically activated, like sepsis.

As our first line of defense, PMN ingest and eventually kill invading pathogens by means of potent antimicrobial agents released during the process of degranulation. As this microbicidal weaponry largely lacks specificity, it can lead to severe tissue damage if not controlled or secluded adequately from surrounding tissue[19-21]. What role(s) ectosomes, which are released from cell activation on, play in PMN's overall functions is largely unknown.

In a first part, the general properties and characteristics of PMN ectosomes will be outlined, with possible implications how ectosomes might implement or modulate PMN functions. The second part will deal with a newly characterised role of ectosomes, namely to act as downmodulators of the, possibly deleterious, inflammatory response initiated by PMN activation, while the last part will focus on the fate of blood-borne PMN ectosomes in circulation. As will be described more in depth, ectosomes activate and bind complement and subsequently adhere to erythrocytes in a complement receptor 1 (CR1)-dependent manner, suggesting that blood-borne ectosomes are behaving, and possibly cleared, similarly to immune complexes.

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## Part I:

## Characterisation and Properties of Ectosomes released by Human Polymorphonuclear Neutrophils


### Abstract

Human neutrophils release vesicles when activated *in vitro* and *in vivo*, in local and systemic inflammation. We have suggested that the presence of these vesicles is due to ectocytosis, defined as the release of rightside-out oriented vesicles expressing a select set of membrane proteins. Herein we have characterised the vesicles released by neutrophils to be ectosomes with specific properties. They contained cytosolic F-actin indicating their outside-out orientation. They bound AnnexinV suggesting that they expose phosphatidyl-serine, similarly to platelet-microparticles. They expressed a subset of cell surface proteins (selectins and integrins, complement regulators, HLA-1, FcγRIII and CD66b but not CD14, FcγRII and CD87). There was no specificity for transmembrane or glycosyl-phosphatidylinositol-linked proteins and unexpectedly L-selectin, known to be cleaved from the surface of activated neutrophils, was present. Ectosomes exposed active enzymes released by neutrophils upon degranulation (matrix metalloproteinase-9, myeloperoxidase, proteinase 3 and elastase). In particular, released myeloperoxidase was able to bind back to ectosomes. The purified complement protein C1q and C1q from serum bound to ectosomes as well. Another aspect of ectosomes was that they became specifically adherent to monocytic and endothelial cells. These observations suggest that neutrophil-derived ectosomes have unique characteristics making them candidates to play roles in inflammation and cell signaling.

## Introduction

Many eukaryotic cells release vesicles spontaneously or under appropriate stimulation. Exosomes are preformed membrane vesicles, which are stored in cellular compartments named multivesicular bodies, and secreted when the multivesicular bodies fuse with the cell-membrane [1]. Many haematopoietic cells including reticulocytes, platelets and leucocytes secrete exosomes. For reticulocytes, exosomes mediate the clearance of obsolete proteins such as the transferrin receptor. Those released by B-lymphocytes bind firmly to follicular dendritic cells and may have the function to present antigens to T-lymphocytes[2]. Exosomes of cytotoxic T-lymphocytes may efficiently deliver cytolytic substances to specific targets[3]. The structure of such exosomes has now been characterised[4].

Beside the release of preformed vesicles, many cells shed small membrane vesicles which are formed directly from the cell membrane[5]. This reaction provides for instance protection against complement attack, since it allows the removal of the C5b-9 attack complex from the cell surface as shown for polymorphonuclear leucocytes (PMNs), oligodendrocytes and even erythrocytes[6-8]. Stein and Luzio coined the term ectocytosis for the release of rightside-out oriented vesicles (ectosomes) from the surface of PMNs attacked by complement[9]. However ectocytosis did not only correspond to the removal of the C5b-9 complex but also to a specific sorting of membrane proteins into the shed ectosomes. Enrichment in cholesterol and diacylglycerol in the ectosome membrane attested for a specific sorting of lipids as well. Hess et al. have provided evidence that when PMNs are stimulated *in vitro* with formyl-methionyl-leucyl-phenylalanine (fMLP) or exposed to calcium ionophores, they release small vesicles in minutes which have many of the properties expected for ectosomes due to complement attack[10]. They expressed a specific set of membrane proteins, including the complement receptor 1 (CR1) and in addition proteases such as myeloperoxidase (MPO) and elastase suggesting that they may function as extracellular antibacterial organelles. Interestingly similar vesicles were isolated from synovial fluids of patients with arthritis,

bronchoalveolar lavage fluids in bacterial pneumonia and skin blisters induced by Cantharidin, all situations in which PMNs are predominant in the exsudate. In addition, electron-microscopy images showed the formation of such vesicles on PMNs by ectocytosis minutes after having added the stimulatory agent.

Various names have been used to describe vesicles released by cells including microparticles, particles, microvesicles and vesicles, probably because the mechanism involved in their release was not always studied in detail[11-21]. A major fraction of microparticles shed by activated platelets corresponds certainly to the definition of ectosomes, as do many others released by monocytes, tumor cells, and fibroblasts[13, 20, 22, 23]. Various functions may be mediated by ectosomes. Mononuclear cell-released microparticles transfer the chemokine receptor CCR5 to cells not expressing it, enabling HIV to infect the recipient cell[15]. The rapid secretion of IL-1 β by THP-1 cells is due to the shedding of ectosomes loaded with IL-1β[14]. Platelet- and monocyte-derived ectosomes induce the coagulation cascade and inflammatory processes by activating endothelial cells[11, 20, 24]. Microparticles released by leucocytes – mainly PMNs – may activate endothelial cells as well[18]. Such microparticles were released by resting leucocytes, but the addition of a stimulus like fMLP increased manifold their shedding. Although the nature of the microparticles was not further analysed, one might speculate that they were ectosomes.

Since vesicles released by activated PMNs may have many biological activities, we set out to further analyse their structure/function characteristics. The data obtained here provide evidence that they are rightside-out oriented, express phosphatidyl-serine, have a very unique pattern of in- and out-shuffled cell-membrane proteins, conserve L-selectin which in contrast is cleaved from the surface of activated PMNs, acquire a specific panel of proteolytic enzymes, bind C1q and can target endothelial as well as monocytic cells.

## Material and Methods

*Antibodies*

All antibodies used were monoclonal mouse anti-human antibodies (mAb). The following mAb were used: phyco-erythrin coupled mAb directed against CD79 and L-selectin/CD62L (Pharmingen / Becton Dickinson), purified mAb against LFA-1/CD11a, Mac-1/CD11b (Pharmingen), CD66b, FcγRIII/CD16 (Immunotech, Marseille, France), myeloperoxidase (MPO), human leucocyte antigen-I (HLA-I; Dako, Glostrup, Denmark), MCP/CD46, CD59 (Sanbio, Netherlands), proteinase 3 (PR3) (CLB, Amsterdam, Netherlands), matrix metalloproteinase-9 (MMP-9) (R&D Systems, Minneapolis, MN), urokinase plasminogen activator receptor (uPAR)/CD87 (Diaclone research, Besançon, France), DAF/CD55 (Biosource, Camarillo, CA), FcγRII/CD32 (NeoMarkers, Fremont, CA), C1q (Quidel, San Diego, CA), FLAG-peptide (IgG1 control directed against the octa-peptide N-Asp Tyr Lys Asp Asp Asp Asp Lys-C) (Eastman Kodak Company, New Haven, CT), and CR1/CD35 (clone 3D9). All non-labeled mAb were biotinylated prior to use (see below).

*Biotinylation of monoclonal antibodies*

Ab were incubated for 1 h at 22°C in the presence of 1 μL biotin-ester (10 mg/mL in DMSO)/50 μg of antibody in sodium borate buffer (0.1 M, pH 8.8), final volume 2 mL. Biotinylated mAb were dialyzed against phosphate buffered saline (PBS) overnight at 4°C in a Slide-A-Lyzer® 10,000 MW cut-off dialysis cassette (Pierce, Rockford, IL). Biotinylated mAb were recovered and concentrated with microsep™ centrifugal concentrators (10,000 MW cut-off, Pall Filtron Corp.) to a final mAb-concentration of 250 μg /mL. Biotinylated mAb were stored at 4°C.

*Isolation and stimulation of PMNs*

PMNs were isolated from fresh buffy coats of normal donors according to the technique described previously[10]. Briefly, a fresh buffy coat obtained from approximately 400 mL of normal donor blood was diluted 1/1 (v/v) with PBS-ethylenediaminetetraacetic acid (EDTA) (2 mM), mixed gently with 0.25 vol of 4% Dextran T500 (Amersham Pharmacia Biotech, Dübendorf, Switzerland), and left for 30 minutes for erythrocyte sedimentation. The leucocyte-rich supernatant (S/N) was aspirated and centrifuged for 10 minutes at 200*g*. The pellet was resuspended for 1 minute in 9 mL of ultrapure water to lyse erythrocytes. Isotonicity was restored by addition of 3 mL of KCl (0.6 M) and 40 mL of NaCl (0.15 M). Cells were then centrifuged 10 minutes at 350*g*, and resuspended in 20 mL of PBS-EDTA (2 mM). This suspension was layered over 20 mL of Ficoll-Hypaque (Sigma, St-Louis, MO) and centrifuged for 30 minutes at 350*g*. The PMN-rich pellet was recovered and washed twice in PBS-EDTA (2 mM). All manipulations were performed at 4°C, thus minimizing PMN-activation.

For stimulation, PMNs ($10^7$ cells/mL) were diluted 1/1 (v/v) in pre-warmed (37°C) RPMI 1640 (Life Technologies, Basel, Switzerland) with fMLP, ionomycin, or PMA (1 μM, 5 μM, 10 nM final concentrations respectively, all from Sigma, St-Louis, MO), and incubated for 20 minutes at 37°C in a water bath. Cell activation was stopped by putting the cell suspensions on ice, and PMNs were removed by centrifugation (two consecutive runs, 20 minutes each, 4000*g* at 4°C). The supernatant (S/N) was concentrated ~50-fold with Centriprep® centrifugal filter devices (10,000 MW cut-off, Millipore Corporation Bedford, MA) and stored in aliquots at -80°C until use.

*Transmission electron microscopy of ectosomes / negative staining*

After ultracentrifugation, ectosomes were resuspended in PBS and then fixed in 1% glutaraldehyd (final concentration) for 20 minutes at room temperature. Ectosomes were then

adsorbed to parlodion-coated copper grids. After washing, samples were stained with 2% uranylacetate before being observed in a Philips Morgani 268 D transmission electron microscope operated at 80kV.

*Fluorescence Activated Cell Scanning (FACScan)-analysis of ectosomes*

Samples were analysed with a FACScan flow cytometer from Becton-Dickinson (Mountain View, CA). The light scatter and fluorescence channels were set at logarithmic gain. An acquisition threshold was set on the forward scatter in order to reduce background-signal. In order to analyse ectosomes by FACScan, concentrated PMN-S/N (see above) was ultracentrifuged for 30 minutes at 160,000*g*/4°C in a SW55ti rotor (Beckman Instruments Inc., Palo Alto, CA). Pelleted ectosomes were washed twice with 0.9% NaCl (B. Braun Medical AG, Emmenbrücke, Switzerland) and incubated at 22°C for 10 minutes in 100 μL 0.9% NaCl/10% normal mouse serum (ultracentrifuged prior to use in order to remove aggregates) (Cedarlane laboratories Ltd., Hornby, Canada). Blocked ectosomes were labeled with 5 μg/mL of biotinylated mAb, or a 1/200 dilution of fluorochrome labeled mAb, in FACScan buffer (PBS, 1% bovine serum albumin (BSA), 10 mM sodium azide) for 10 minutes at 22°C. Ectosomes were then pelleted by ultracentrifugation (30 minutes, 160,000*g*/4°C), and washed twice in FACScan buffer. Bound biotinylated antibodies were revealed with 2 μL streptavidin-phyco-erythrin/100 μL FACScan buffer (incubated for 10 minutes at 22°C). Labeled ectosomes were then resuspended in 200 μL 0.9% NaCl and analysed on a FACScan. Forward (FSC) and side scatter (SSC) as well as fluorescence channels (FL1 and FL2) were set on logarithmic scales.

*Membrane labeling of ectosomes*

An amphiphilic cell linker dye kit (PKH67, Sigma, St-Louis, MO) was used, following the labeling procedure provided by the manufacturer. Briefly, ectosomes resuspended in 200 μL

Diluent C were incubated with 200 μL diluent C/dye solution (dye diluted 1/200) for 30 seconds at 22°C, with gentle shaking. 1 mL RPMI 1640 (without phenol red) was added to stop the reaction. Labeled ectosomes were separated from the remaining unbound dye by ultracentrifugation (45 minutes, 160,000*g*/4°C) and washed with 0.9% NaCl. In order to avoid capping of Ab with dye, membrane-labeling was made prior to antibody-binding steps.

*Binding of annexin V to ectosomes*

Ectosomes were isolated from concentrated PMN-S/N and washed as described above. Washed ectosomes were resuspended in either 100 μL 0.9% NaCl, or AnV binding buffer (10 mM Hepes/NaOH, 140 mM NaCl, pH 7.4) with or without 2.5 mM $CaCl_2$. 2μL AnV-FITC or AnV-biotin were added to each reaction. After incubation for 10 minutes/22°C ectosomes were washed twice in their respective buffer. When using AnV-biotin, a second step with SA-PE was performed as described above. Ectosomes where then resuspended in 200 μL 0.9% NaCl and analysed by FACScan.

*Western blot analysis/zymography of matrix metalloproteinase-9*

Western blotting and zymography were performed after separation of ectosomal proteins by 7.5% sodium-dodecylsulfate polyacrylamide-gel-electrophoresis (SDS-PAGE). For zymography, gels were supplemented with gelatin (1 mg/mL) (Sigma, St-Louis, MO). For zymography, gels were incubated in zymography buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) overnight at 37°C and stained in Brilliant Blue. Recombinant active MMP-9 was used as positive control (Oncogene, Cambridge, MA; 0.5 μg/lane). For Western blots, gels were transferred onto nitrocellulose membranes, which were blocked overnight at 4°C in PBS/6% skimmed milk powder. Membranes were then washed briefly in PBS/0.01% Tween 20 and incubated with anti-MMP-9 antibodies (0.5 μg/mL in PBS/1% BSA) for 1 hour at

22°C. Membranes were then washed three times for 10 minutes in PBS/0.01% Tween 20 and bound mAb detected using goat anti-mouse antibodies. Finally, membranes were incubated for 45 minutes in horse-radish-peroxidase coupled streptavidin diluted in PBS-BSA. Detection was by enhanced chemi-luminescence (ECL kit, Amersham Pharmacia Biotech) with BioMax™ films (Kodak, Rochester, NY).

Alternatively, to check for the presence of MMP-9 inside ectosomes we probed whole ectosomes as well as sonicated ectosomes with an EnzChek™ Gelatinase/Collagenase Assay kit (Molecular Probes, Eugene, OR), following the manufacturer's indications.

*MPO and elastase activity assays*

Enzymatic activity of MPO was determined in a colorimetric assay using whole ectosomes, where 50 μL of orthophenyldiamine-solution (2 μg in 5 mL citrate-phosphate buffer, pH 5, with 2 μL of 35% $H_2O_2$) were incubated with 100 μL of the sample to be tested. The reaction was stopped using 2.5 M $H_2SO_4$, and the absorbance measured with a microplate reader (Thermo Max, Molecular Devices, Menlo Park, CA) at 490 nm[25]. Enzymatic activity of elastase was analysed using an EnzChek™ Elastase Assay kit (Molecular Probes, Eugene, OR). Where indicated elafin (a specific inhibitor of elastase, kindly provided by Dr. Dougie Paterson, Zeneca Pharmaceuticals, GB) was used at a concentration of 30 μg/mL. To check for the presence of elastase inside ectosomes we assessed elastase activities of whole ectosomes as well as sonicated ectosomes with this kit.

*Detection of ectosomes in whole blood*

Fresh citrate anti-coagulated blood was obtained from normal blood donors. For activation, 5 mL of whole blood was incubated with 1 μM fMLP for 20 minutes at 37°C. Immediately after activation, cells were removed by centrifugation (10 minutes, 1000*g*, 25°C) and S/N stored at -80°C until use.

For isolation of blood-borne vesicles, including ectosomes, plasma from activated whole blood was ultracentrifuged as for PMN-S/N. Vesicles were then stained with AnV and mAb against CD66b. Fluorescence of vesicles was detected by FACScan-analysis.

*Binding of exogenous MPO to MPO-deficient ectosomes*

MPO-deficient as well as normal PMNs were isolated and activated as described above. Ectosome-free normal PMN-S/N was kept and stored until further use at 4°C. After a washing step with 0.9% NaCl, MPO-deficient ectosomes were incubated 10 minutes at 22°C with 200 μL of ectosome-free normal PMN-S/N, 100 ng of purified MPO added to 200 μL PBS, or 200 μL PBS. Ectosomes from normal donors were incubated with PBS only. Ectosomes were then washed twice and stained for MPO with biotinylated mAb anti-human MPO, followed by streptavidin-phyco-erythrin as described above. Labeled ectosomes were analysed by FACScan-analysis.

*Binding of C1q to ectosomes*

Ectosomes were isolated as described above. Ectosomes were then ultracentrifuged and incubated, after gentle resuspension, for 30 minutes at 4°C in 100μL of 0.9% NaCl alone or 0.9% NaCl supplemented with either 1μg of purified C1q (Calbiochem, La Jolla, CA), normal human serum (1/10 final dilution) or heat-inactivated normal human serum (1/10 final dilution; heat inactivation: 30 minutes at 56°C). The binding of C1q was then detected using a biotinylated anti-C1q antibody and streptavidin-phyco-erythrin, as described for all other FACScan-analyses of ectosomes.

*Cell cultures*

All cultures were maintained in 5% $CO_2$ at 37°C and medium replaced every second day. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego,

CA) and cultured in medium consisting of M199 supplemented with 20% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL heparin and 50 µg/mL endothelial cell growth supplement (Sigma, St-Louis, MO). Cells were split by trypsinization (0.025% trypsin solution) when confluent. THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine.

*Binding assays of ectosomes to human erythrocytes, endothelial and phagocytic cells*

Confocal microscopy: Ectosomes were prepared and labeled as described above. HUVEC (of the second to fifth passage) were cultured on gelatin-coated culture slides. THP-1 cells were cultured on uncoated culture slides for three days in the presence of 160 nM PMA for differentiation into phagocytic cells. HUVEC and THP-1 cells were then incubated with labeled ectosomes for 30 minutes washed three times with medium and analysed (Zeiss microscope). For these experiments, ectosomes were counted by FACS analyses, gating on CD66b positive events. Incubations were performed using a cell to ectosome ratio of 1:10.

FACScan analysis: Erythrocytes were isolated from freshly drawn EDTA-anticoagulated blood from a normal donor by dextran sedimentation. Labeled ectosomes were incubated for 30 minutes at 37°C with the different cell types. Binding assays were performed using $10^6$ erythrocytes/mL in a final volume of 100 µL. The same concentration was used for HUVEC and THP-1 cells. For blocking experiments, prior to adding labeled ectosomes, cells were incubated for 10 minutes with the indicated proportions of unlabeled ectosomes. Cells were then washed, resuspended and analysed by FACScan. As mentioned above, incubations were performed using a cell to ectosome ratio of 1:10.

*Two-dimensional gel electrophoresis and data analysis*

A commercial sigmoidal immobilised pH gradient (IPG) going from pH 3.5 to 10.0 was used for first dimensional separation. After equilibration, IPG gel strips were transferred for the second dimension onto vertical gradient slab gels (9-16% T, 2.6% C) and run with the Laemmli-SDS-discontinuous system. Protein detection was done using a sensitive ammoniacal silver stain or Coomassie Brilliant Blue R-250 (0.1% w/v) and methanol (50% v/v) for 30 minutes. Destaining was done in a solution containing methanol (40% v/v) and acetic acid (10% v/v). Gels were then scanned using a laser densitometer (APB, Uppsalla). The 2-D PAGE image computer analysis was carried out using the MELANIE 3 software package (GeneBio, Geneva, CH). Optical density, area and volume were computed and related to protein concentration. Also, the relative optical density and relative volume was calculated in order to correct for differences in gel-staining. Differentially expressed proteins were determined by differential analysis and significance in differences was tested using Student T-test. A $p<0.05$ was considered significant.

*Protein identification by tandem mass spectrometry (MS)*

Coomassie blue 2-DE spots were excised from the 2-D gel and destained with 100 µL of 30% acetonitrile in 50 mM ammonium bicarbonate at 37°C for 45 minutes. The S/N was discarded and the gel spots dried in a SpeedVac for 30 minutes. The gel spots were rehydrated with 10 µL of a solution containing 6.25 ng/µL of porcine trypsin in 50 mM ammonium bicarbonate buffer for 45 minutes on ice. Digestion was carried out overnight at 37°C. Resulting peptides were extracted at 22°C by two sequential extraction steps of 20 minutes. The first extraction is performed with 20 µL of 1% TFA and the second one with 20 µL of 0.1% TFA in 50% acetonitrile. The combined extracts were concentrated in a SpeedVac to approximately 2 µL, redissolved in 30-40 µL of 0.1% TFA and dried again down to approximately 2 µL. Then 5 µL of a 0.1% HCOOH solution was added. 5 µL of the peptide extract were loaded onto a 75

µm diameter and 10 cm long C18 micro-column. Peptides were eluted at 400 nL/minute by a linear gradient of acetonitrile in 0.1% HCOOH and directly analysed by nanoESI-MS/MS conducted on a Q-TOF mass spectrometer from Micromass (Manchester, UK). MS/MS spectra were acquired by automatic switching between MS and MS/MS mode by the instrument. Acquired fragment ion spectra were searched by Mascot against databases such as SWISS-PROT, TrEmbl or ESTs databases.

## Results

*Qualitative Analysis of ectosome-preparations*

Ectosome-preparations were tested for their quality and purity by electron microscopy. As shown in Figure 1 a), ectosome-preparations did not present any major impurities. Figures 1 b), c) and d) represent higher magnification pictures of ectosomes, showing again the absence of any microparticles other than ectosomes in our preparations. The size heterogeneity of ectosomes is documented in Figure 1 b), which shows ectosomes of different sizes, located next to each other (arrows).

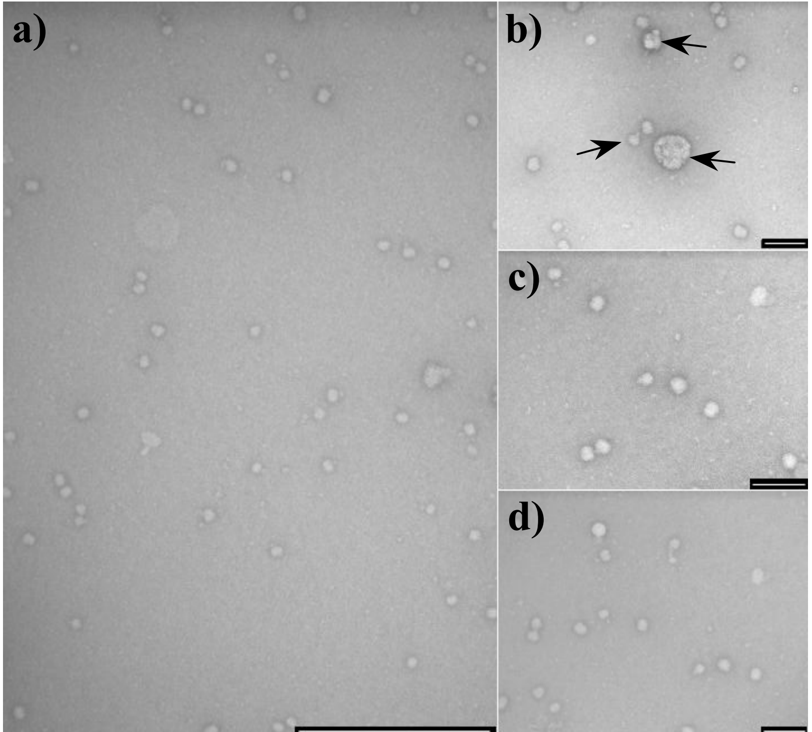


**Figure 1: Electron microscopy of ectosomes**

a) Overview on the purity of ectosome-preparations used for analyses. Size bar: 500 nm. b), c) and d) higher magnification picture of ectosomes. All size bars: 100 nm.

*Flow-cytometry-based detection of PMN-derived vesicles*

The vesicles released by PMNs activated with 1µM fMLP were recovered after 20 min of incubation at 37°C by differential centrifugation. First PMNs were pelleted by centrifugation

at 4000g for 2X20 min, the supernatant containing the vesicles was then ultracentrifuged at 160,000g for 30 min. The pelleted vesicles were resuspended and could be dissociated by gentle mixing in 0.9% NaCl. By flow-cytometry such vesicles were found to form a distinct FSC/SSC-population. The size heterogeneity was reflected by a simultaneous scattering towards higher FSC and SSC values. A typical FSC/SSC plot of purified vesicles and the standard gate as set for their analysis is shown in Figure 2A. The detection of synthetic fluorescent beads of 200 nm diameter within the gate set for vesicles confirmed their approximate size (of range 50 to 200 nm)[10] (data not shown).

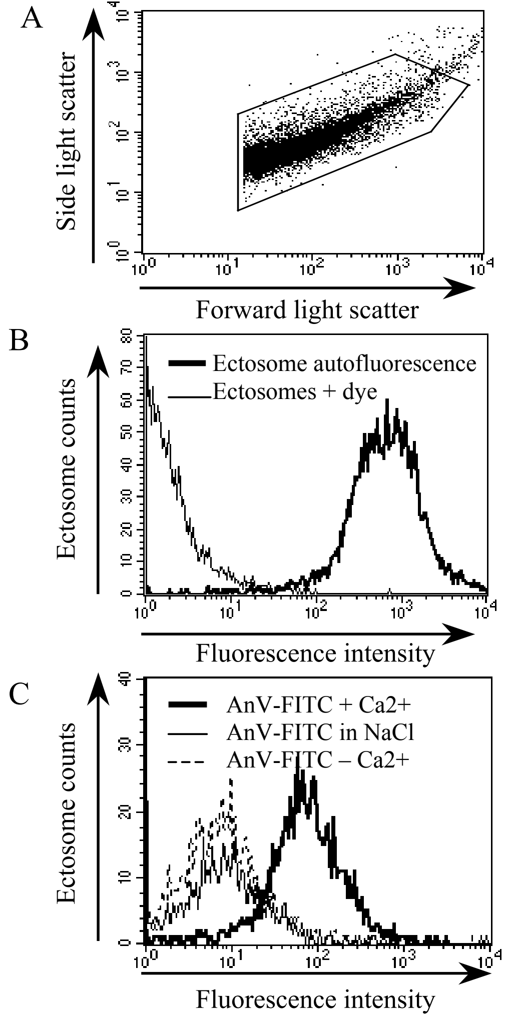


**Figure 2: Detection of ectosomes by FACScan**

Scatter dot plot of ectosomes derived from activated normal PMNs. The gate (R1) contains the majority of ectosomes. (B) Fluorescence histogram of ectosomes before (thin line) and after (thick line) membrane labeling. (C) Fluorescence histogram of AnV-FITC stained ectosomes. Ectosomes were incubated with AnV in binding buffer (thick line), calcium depleted binding buffer (dotted line), or 0.9% NaCl solution (thin line).

Using an amphiphilic, membrane-interchelating dye, particles within the above-defined gate uniformly acquired fluorescence (Figure 2B). Interestingly the intensity of the fluorescence correlated directly with both FSC and SSC, indicating that larger vesicles had taken up more dye (data not shown).

Under many circumstances vesicles released by activated cells express phosphatidyl serine, a marker for the loss of lipid-membrane assymetry[5]. By flow cytometry we could show that annexin V (AnV), which binds PS in a calcium-dependent manner, bound to the vesicles (Figure 2C). This binding was prevented by depleting calcium from the buffer or using a saline solution deprived of calcium. Thus the vesicles exposed negatively charged phospholipids, very likely to be phosphatidyl-serine. An AnV/membrane-dye double-labeling indicated clearly that AnV binding was present on all lipid-expressing vesicles, with the binding of AnV correlating directly with the size of the vesicles.

*Protein expression by PMN-derived vesicles*

We then set out to analyse the proteins expressed by these vesicles by two-dimensional gel-electrophoresis (proteome) (Fig 3A). We identified actin and annexin III as two of the major proteins present by comparing 2-DE patterns of vesicles with a 2-DE library (computer-aided) and by sequencing (Fig 3A-B). Actin is a cytosolic protein, which would confirm that the vesicles form by budding from the cell-membrane and incorporate some cytoskeletal components of the cytosol. By flow-cytometry phalloidin, which binds F-actin, was used to confirm the presence of actin in the vesicles. Interestingly phalloidin did react with permeabilised vesicles only, and the intensity of staining was proportional to the concentration of the detergent used (Fig 3C). By contrast, intact vesicles did not bind phalloidin. Thus actin was only present inside the vesicles, suggesting that they were rightside-out oriented. Permeabilisation did not alter the binding of mAb specific for proteins

present on the surface of ectosomes (described below), thereby ruling out the possibility that the detection of actin was due to phalloidin trapped inside ectosomes.

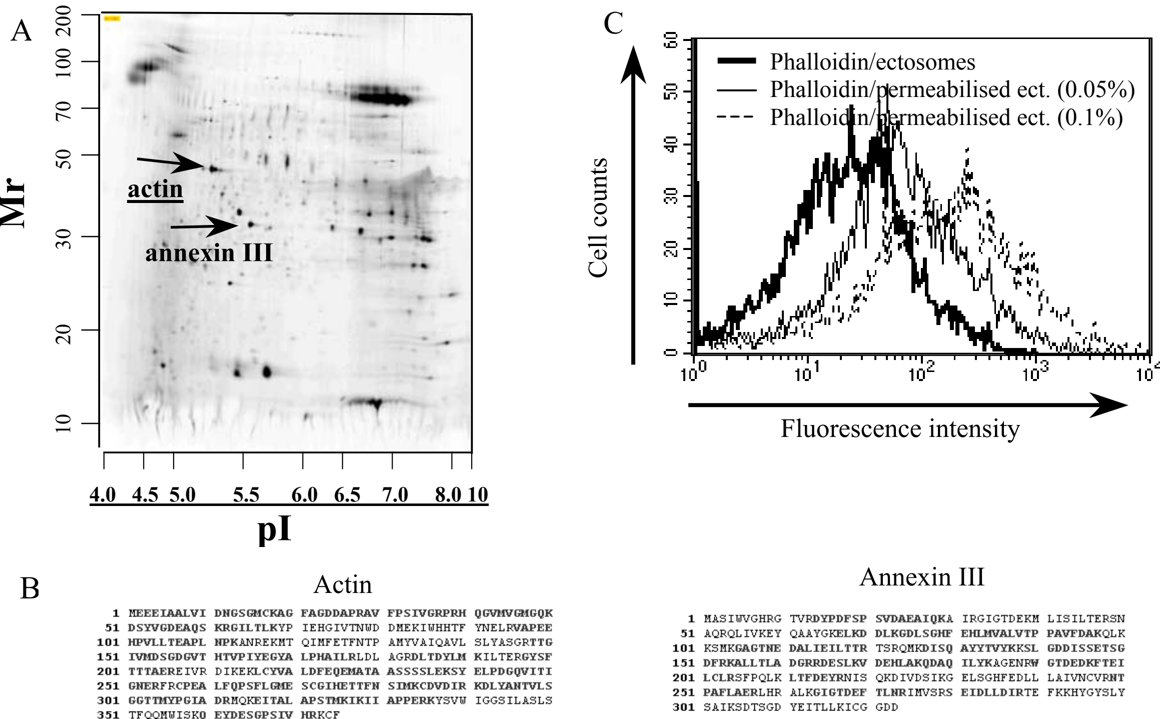


**Figure 3: Detection of ectosome-associated annexin III and intraectosomal actin**

(A) 2D-GE of ectosomal proteins. By mass spectrometry, protein spots indicated by arrows were identified as actin and annexin III, as indicated. (B) Trypsin-digested excised spots were analyzed by tandem mass spectrometry and ion spectra interpreted with the Mascot database search. Peptide sequences matching the database sequence are shown in bold letters. For actin and annexin III, 21 and 16 matching peptides were obtained with sequence recovery percentages of 69% and 52%, respectively. (C) Binding of phalloidin to ectosomes was analyzed in permeabilizing buffers containing increasing concentrations of detergent (thin line: 0.05% Nonidet®P40, dotted line 0.1% Nonidet®P40). The control experiment was done using the same buffer without detergent (thick line).

In parallel we analysed the expression of proteins on the surface of the vesicles by using mAb against different PMN-proteins using purified PMNs as controls. With this approach, many transmembrane [HLA-I, CD35 (CR1), CD11a, CD11b, CD62L (L-selectin), CD46 (MCP)] and GPI-anchored proteins [DAF, CD59, CD66b, CD16 (FcγRIII)] were identified on the vesicles (Table 1). Of particular interest was the presence of L-selectin, which is cleaved off PMNs at the time of cell activation, but which is clearly lost from PMNs by vesiculation as well. In addition to proteins derived from the PMN cell-surface, vesicles also expressed

| *PMN-proteins not detected on ectosomes* | *PMN-proteins detected on ectosomes* |
|---|---|
| Example shown: uPAR/CD87 | Example shown: HLA-1 |
| Other proteins not detected:<br><br><u>Receptors:</u> CD14 and FcγRIII/CD32. | Other proteins detected:<br><br><u>Receptors:</u> CR1/CD35, LFA-1/CD11a, Mac-1/CD11b, FcγRIII/CD16 and L-selectin.<br><u>Enzymes:</u> Myeloperoxidase, Elastase, MMP-9 and PR3.<br><u>Complement proteins:</u> MCP/CD46 and CD59.<br><u>Other:</u> CD66b |

**Table 1: Summary of PMN-proteins found on ectosomes by FACScan analysis.**

In FACS histograms, dotted line represents fluorescences of ectosomes (in R1) incubated with isotype-control mAb and thick line represents fluorescences of ectosomes (in R1) incubated with mAb against the mentioned antigen.

| | Ectosomes | Cell membrane | Azurophilic granules | Specific granules | Gelatinase granules | Secretory vesicles | Protein type |
|---|---|---|---|---|---|---|---|
| HLA-1 | + | + | - | - | - | - | TM |
| CR1/CD35 | + | + | - | - | - | + | TM |
| CD11a | + | + | - | - | - | - | TM |
| CD11b | + | + | - | + | + | + | TM |
| L-selectin | + | + | - | - | - | - | TM |
| MCP/CD46 | + | + | - | - | - | - | TM |
| CD66b | + | + | - | + | - | - | GPI |
| DAF/CD55 | + | + | - | - | - | - | GPI |
| FcγRIII/CD16 | + | + | - | - | - | + | GPI |
| CD59 | + | + | - | - | - | - | GPI |
| uPAR/CD87 | - | + | - | + | + | + | GPI |
| CD14 | - | + | - | - | - | + | GPI |
| FcγRII/CD32 | - | + | - | - | - | - | TM |
| MPO | + | - | + | - | - | - | soluble |
| elastase | + | - | + | - | - | - | soluble |
| proteinase 3 | + | - | + | + | - | + | soluble |
| MMP-9 | + | - | - | + | + | - | soluble |

**Table 2: Cellular localisation of proteins analysed for their presence on ectosomes.**

Legend: +: present, -: absent, TM: transmembrane anchored protein, GPI: glycosyl-phosphatidylinositol linked protein.

elastase, proteinase 3 (PR3), MPO and MMP-9, all stored within granules of resting PMNs (Table 2), with some enzymes producing a more intense staining than others. Furthermore, by double-labeling we could confirm that the fluorescence for MPO was directly proportional to the fluorescence obtained after membrane-staining (amphiphilic dye; Fig 4C). Electronic compensation during acquisition of double-staining data was used to reduce the spectral overlap of both fluorochromes. An identical proportional increase was seen when testing CD46 (MCP) versus AnV (Fig 4B).

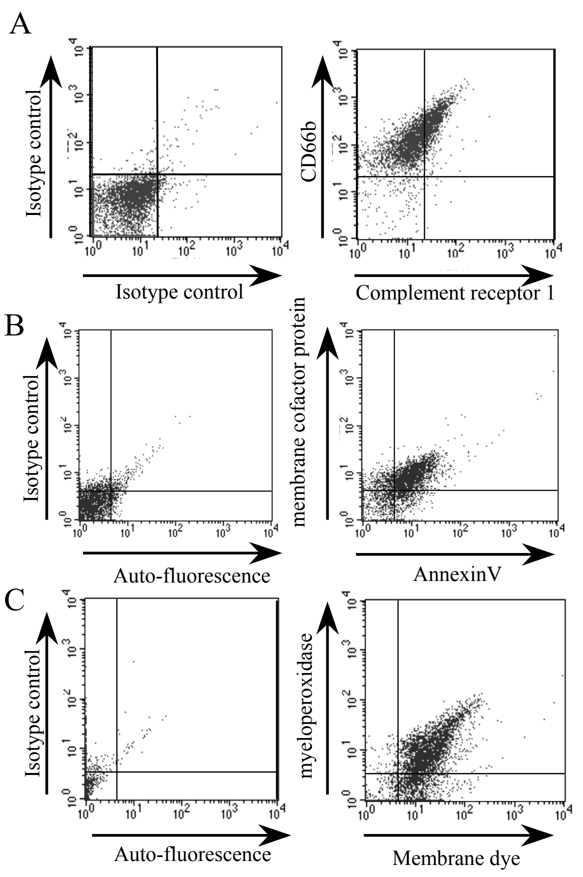


**Figure 4: FACScan analysis of protein/protein and membrane/protein double-labeled ectosomes**

(A) Isotype-control double-labeled ectosomes are shown on the left, CD35/CD66b double-labeled ectosomes on the right. (B) Isotype-control labeled ectosomes are shown on the left, AnV/CD46 double-labeled ectosomes on the right. (C) Isotype-control labeled ectosomes are shown on the left, membrane dye/MPO double-labeled ectosomes on the right. All data represent fluorescence intensities of ectosomes gated in R1.

Other double-labeling studies suggested that this was a general rule for ectosomal proteins, i.e. the intensity of fluorescence being proportional to membrane-staining. Indeed we tested both CD16 (FcγRIII) and elastase versus AnV and obtained similar results (data not shown). In fact, every double-labeling suggested the same general rule: all staining-intensities correlated positively, even when two membrane proteins were plotted versus each other (e.g. CR1/CD66b, Figure 4A). In addition, the correlation between signal intensities of detected

proteins and/or membrane confirmed that the detection of protein was limited by the size of the vesicles. Even the smallest vesicles were shifted by the specific label, although this shift was sometimes minimal (membrane-dye single-positive; e.g. Figure 4C, right plot, lower right hand side quadrant). Therefore, large vesicles are likely to be biologically more active. Furthermore, we could not detect different populations of vesicles, i.e. we found no discrepancies between all the markers used. By contrast, the vesicles (even when gating on their larger fraction only) did not express detectable amounts of CD14, CD32 (FcγRII) and CD87 (urokinase plasminogen activator receptor, uPAR), while the same molecules could clearly be detected on PMNs (data not shown).

Taken together, the data indicated that the vesicles have all the hallmarks of ectosomes as described by Stein and Luzio[9]. However, one additional aspect was uncovered in the sense that ectosomes may acquire enzymes, which are released by degranulation of PMNs at the time of their activation.

*Binding of MPO to ectosomes*

Taking advantage of an MPO-deficient individual the mechanism by which MPO (stored within the azurophilic granules of resting PMNs) may become translocated to the membrane of ectosomes was studied. Firstly, purified MPO in normal buffer (PBS) bound to MPO-deficient ectosomes in a dose-dependent and saturating manner (data not shown). The fluorescence intensity reflecting the presence of MPO on ectosomes reached the level of normal ectosomes (Figure 5A-B). Secondly, using a more physiological assay, MPO from ectosome-free S/N from degranulated PMNs of normal donors (containing soluble MPO) bound to a similar extent to MPO-deficient ectosomes (Figure 5B), indicating that at least a fraction of MPO rebinds to the surface of the ectosomes.

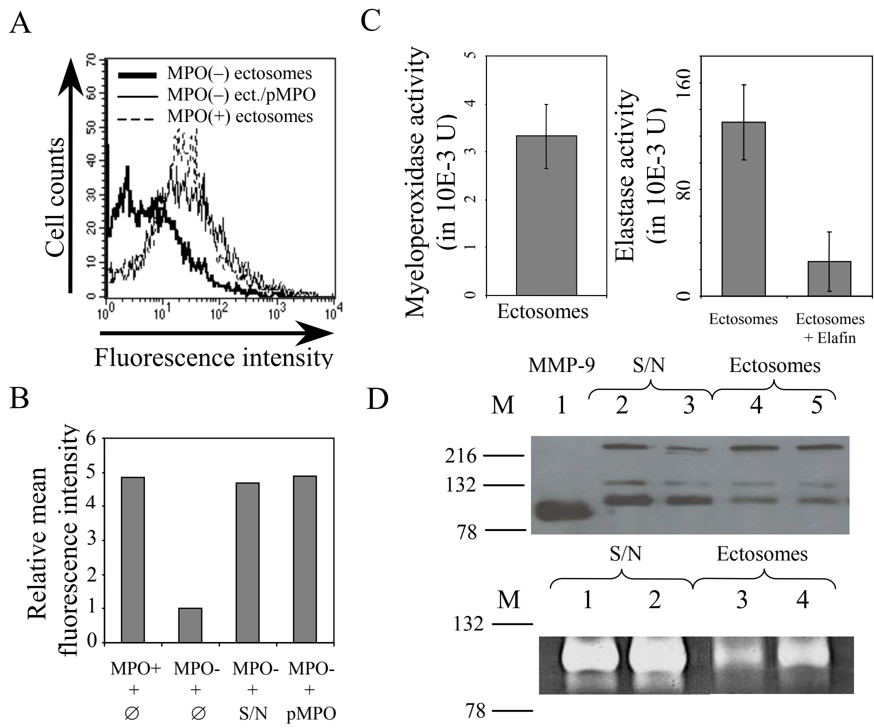


**Figure 5: Ectosomes expose active MPO, PR3 and MMP-9 at their surface**

(A) FACScan-analysis of MPO-expression on ectosomes derived from a normal donor (dotted line) and an MPO-deficient individual incubated with purified MPO (thin line) or without MPO (thick line) (gate R1). (B) Relative mean fluorescence intensities of ectosomes derived from a normal donor (column 1) and an MPO-deficient donor (column 2). Ectosomes derived from an MPO-deficient individual were incubated with MPO-containing, ectosome-free S/N from normal degranulated PMNs (column 3), or with purified MPO (column 4). The arbitrary value 1 was set for MPO-deficient ectosomes. (C), left hand panel, Ectosome-associated MPO activity expressed in units (U) released by $10^6$ activated PMNs within 20 minutes. Data represents results from 2 independent experiments. (C), right hand panel, Ectosome-associated elastase activity in presence (column 1)/absence (column 2) of elafin. Activity is expressed in U released by $10^6$ activated PMNs within 20 minutes, data represents results from 4 independent experiments for each assay. (D) Western blot analysis of ectosomes, ectosome-free S/N and rMMP-9. Each lane corresponds to an independently prepared sample. (B) MMP-9 activity of ectosomes and ectosome-free S/N as assessed by gelatin zymography. Each lane represents an independently prepared sample.

*Functional analysis of ectosomal enzymes*

The next question was to see whether the enzymes fixed on ectosomes remained functionally active. In an assay for MPO-mediated catabolism of hydrogen peroxide, whole ectosomes were found to be capable of oxidizing ortho-phenylenediamine (Figure 5C). This also held true for ectosomes that acquired MPO from the soluble phase (data not shown).

The presence of matrix metalloproteinase-9 (MMP-9) was confirmed by Western blot of ectosomal proteins (Fig 5D, upper panel). The migration of the monomeric form of MMP-9 from ectosomes was slightly different from the control (active) MMP-9, suggesting that it was in its pro-form. We obtained three distinct bands of apparent molecular weights 92kDa, 130 kDa and 220 kDa, which seem to correspond to all three storage-forms of MMP-9 in PMNs, namely pro-MMP-9 monomer, the MMP-9/lipocalin heterodimer and the MMP-9 homodimer, respectively. All forms of MMP-9 were then shown to be enzymatically active by their capacity to degrade gelatin in a zymographic assay (Fig 5D, lower panel; only monomeric MMP-9 shown).

Whole ectosomes had elastolytic activity, which was in part inhibitable by elafin, a specific inhibitor of elastase/PR3 (Fig 5C). Elastase as well as MMP-9 activities remained unchanged when ectosomes were first lysed by sonication (data not shown). These data suggest that elastase/PR3 and MMP-9 were present almost exclusively on the surface of ectosomes and not stored inside ectosomes.

*Ectosomes released by PMNs can be identified in whole blood*

An important aspect was to see whether the reactions observed with purified PMNs would be similar in a more physiological environment, and would correspond to the activation of circulating PMNs as described in several clinical situations[26-28]. Therefore, PMN-activation in whole-blood was simulated *in vitro*, and a system to specifically trace PMN-derived ectosomes was established. fMLP was added to citrate-anticoagulated blood and incubated for 20 minutes at 37°C. Thereafter cells were removed and the S/N applied to ultracentrifugation. The pellet was redissolved and analysed by FACScan. A typical FSC/SSC plot of microparticles obtained from activated whole blood is shown in Figure 6A. Phosphatidyl-serine expressing membrane-particles were traced by staining with AnV, and PMN-derived ectosomes were distinguished from non-PMN-derived microparticles by tracing

the PMN-specific protein CD66b. As shown in Figure 6B, a majority of particles obtained from the S/N of activated whole-blood was binding AnV (≈70%). In contrast, only a sub-population of AnV-binding particles also expressed CD66b (≈13% of the total events or ≈20% of AnV positive events; Figure 6C). The AnV/CD66b-double-positive population (i.e. PMN-derived ectosomes) localised in a distinct region of the FSC/SSC plot (Figure 6C), comparable to the one delimited by ectosomes obtained from isolated PMNs (Figure 2A). This gate was devoid of CD66b negative, i.e. non-PMN-derived particles, indicating that PMN-derived ectosomes exhibit particular biophysical characteristics distinguishing them from other blood-borne microparticles by FACScan. CD66b-positive (PMN-derived), as well as single-, AnV-positive ectosomes could be detected using the same technique in control plasma (without fMLP), however, in significantly lower numbers than in the supernatant of blood stimulated with fMLP (data not shown). This finding is consistent with previous reports describing the presence of ectosomes in the circulation of healthy individuals[19, 29].

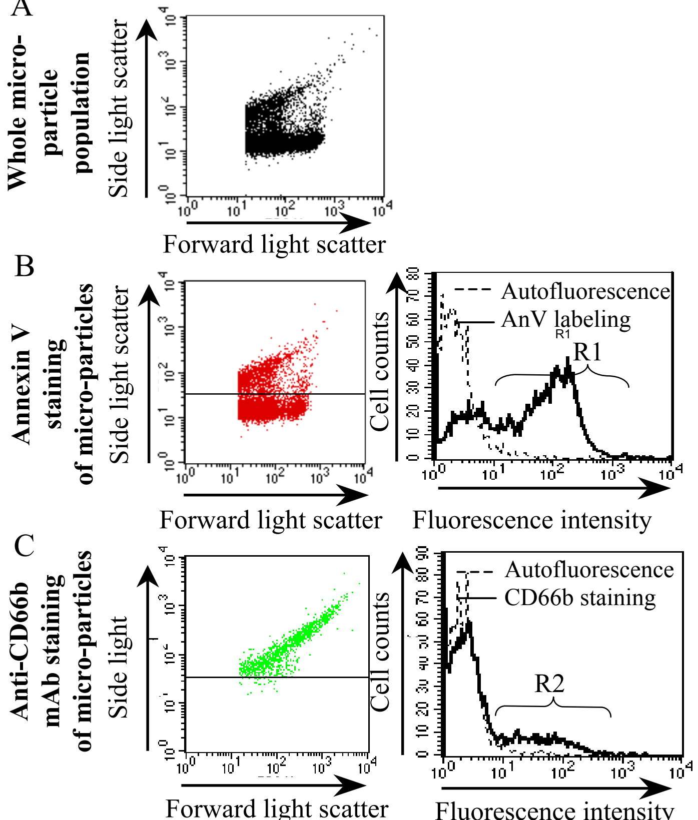


**Figure 6: Detection of ectosomes in whole blood**

(A) Scatter dot plot of total micro-particles (MP) derived from fMLP stimulated whole blood. (B) The dotted and filled lines in the histogram correspond to unstained and AnV-FITC stained blood MP, respectively. Gate R1 includes all AnV-positive MP. The scatter dot plot represents FSC/SSC characteristics of AnV-positive MP only (gated on R1). (C) The dotted and filled lines in the histogram correspond to unstained and anti-CD66b stained MP, respectively. Gate R2 includes all CD66b-positive MP. The scatter dot plot represents FSC/SSC characteristics of CD66b positive MP only (i.e. ectosomes, gate R2).

*Ectosomes bind to the surface of selective cell-types*

Since ectosomes express various adhesion proteins including L-selectin, we tested for their capacity to bind to endothelial cells (HUVEC) and a human monocytic cell line (THP-1) using human erythrocytes as controls. Ectosomes were labeled with membrane dye and incubated with the aforementioned cell-types. Thereafter cells were washed extensively in order to release weakly associated ectosomes. As assessed by FACScan analysis, both HUVEC and THP-1 cells bound ectosomes in a dose dependent, and saturable manner. Competition for binding-sites on HUVEC and THP-1 cells was revealed by their displacement with unlabeled ectosomes (Figures 7A-B), a displacement which was concentration dependent. No binding of ectosomes to erythrocytes was observed (Figure 7E). This was in contrast to liposomes [70% PS, 30 % phosphatidyl-choline (PC)], which were found to bind nonspecifically to the cell-surface of all three cell-types (data not shown). Using confocal microscopy, fluorescent ectosomes were visualised as discrete spots on the cell-surface of both HUVEC and THP-1 cells, confirming the data obtained by FACScan (Figure 7C-D).

*Binding of C1q to the surface of ectosomes*

The binding of C1q to apoptotic cells/bodies has been shown to be one of the central reactions which allows the efficient removal of apoptotic cells by phagocytes. To see whether C1q would also bind to ectosomes, we incubated them for 30 minutes at 4°C with or without purified C1q (10µg/ml final concentration) in 0.9% NaCl, normal human serum or heat-inactivated normal human serum (1/10 final dilutions). C1q was detected on ectosomes after incubation with purified C1q and more interestingly after incubation with whole serum, in which C1q is associated in the macromolecular C1 complex (Figure 8). No signal was detected in the control heat inactivated serum or in the absence of C1q.

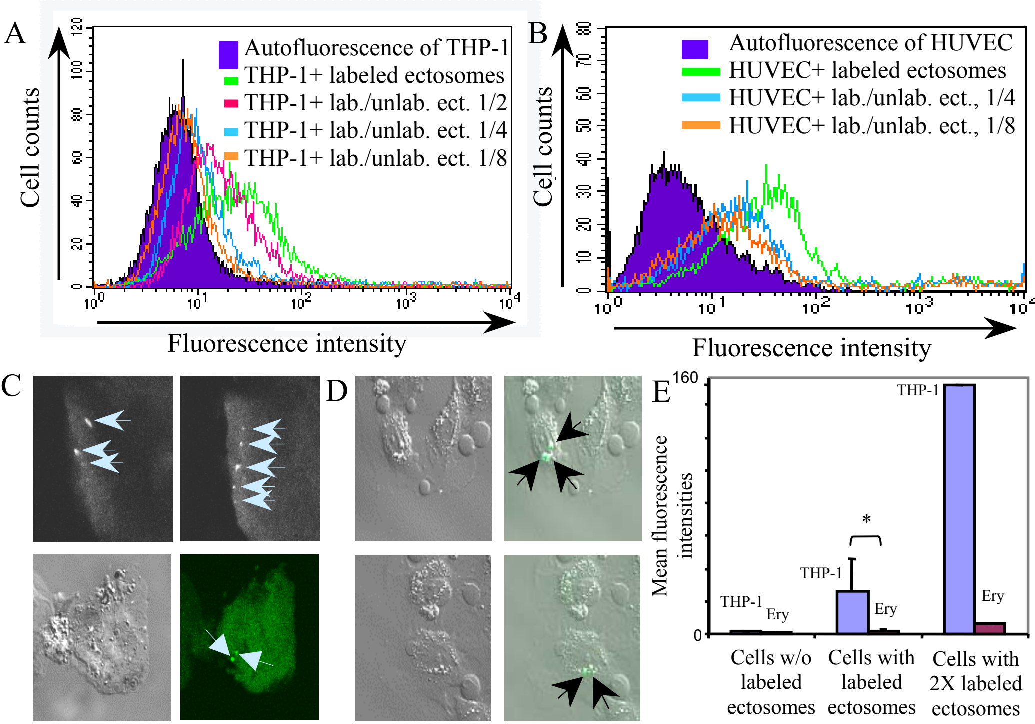


**Figure 7: Binding of ectosomes to selective cell types: THP-1, HUVEC but not erythrocytes.**

(A) Histogram plot of THP-1 cells incubated with ectosomes labeled with membrane-dye. THP-1 cells were incubated with non labeled ectosomes prior to being incubated with labeled ectosomes. Filled violet corresponds to auto-fluorescence of THP-1 cells, the green line represents fluorescence of cells incubated with labeled ectosomes in absence of non-labeled ectosomes, red, blue and orange lines represent THP-1 cells incubated with non-labeled/labeled ectosomes in a ratio 2/1, 4/1 and 8/1, respectively. (B) Histogram plot of HUVEC incubated with fluorescent ectosomes and analysed by FACScan. HUVEC were pre-blocked with various amounts of non-labeled ectosomes and subsequently incubated with a similar amount of labeled ectosomes. Filled violet corresponds to auto-fluorescence of HUVEC, the green line represents fluorescence of cells incubated with labeled ectosomes in absence of non-labeled ectosomes; blue and orange lines represent HUVEC incubated with non-labeled/labeled ectosomes in a ratio of 4/1 and 8/1, respectively. (C) Confocal microscopy of fluorescent ectosomes bound to THP-1 cells. Upper panels, pictures of a single THP-1 cell after incubation with fluorescent ectosomes at two different focal levels. Lower panels, pictures of a THP-1 cell after incubation with fluorescent ectosomes in phase contrast (left) as well as in fluorescence modus (right). Bound fluorescent ectosomes are marked with arrows. (D) Confocal fluorescence microscopy of HUVEC after incubation with labeled ectosomes. On the left HUVEC are shown in phase contrast, on the right in the fluorescence modus. Bound ectosomes are indicated with black arrows. (E) Comparison of mean fluorescence intensities (expressed in relative fluorescence units) of either THP-1 cells (left columns, THP-1) or erythrocytes (right columns, Ery) incubated 30 minutes without (data series 1) or with increasing amounts (data series 2-3) of labeled ectosomes (* $P = 0.16$ with student's t-test). Experiments were carried out in parallel with same amounts/concentration of cells in each assay. Results are the mean ± SEM of two experiments. Data shown in all 5 parts of this figure were obtained with different ectosome preparations.

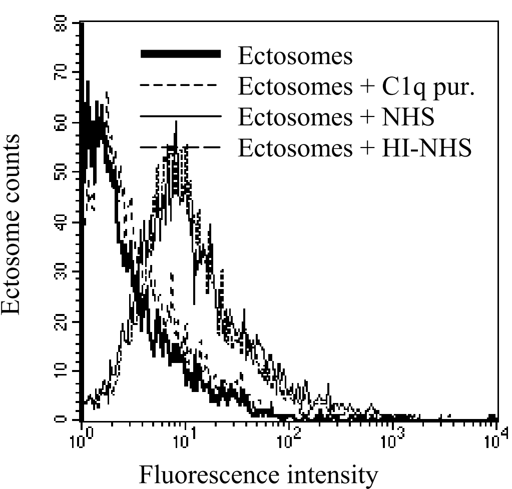


**Figure 8: Binding of C1q to ectosomes**

FACScan histogram of ectosomes incubated for 30 minutes at 4°C in the presence or absence of C1q and subsequently probed with a biotinylated anti-C1q mAb and streptavidin-phycoerythrin. The bold and short dotted lines represent ectosomes incubated in 0.9% NaCl in the absence and presence of 10µg/mL purified C1q, respectively. The thin and long dotted lines represent ectosomes incubated in 0.9% NaCl in the presence of 10% (final dilution) normal human serum and heat-inactivated normal human serum, respectively.

## Discussion

In the present studies we analysed biochemical and immunological characteristics of vesicles released by activated PMNs. The main findings were that these vesicles corresponded in many ways to ectosomes as described by Stein et al., since they expressed a selected set of membrane proteins, and were rightside-out oriented vesicles containing cytoplasmic proteins[9]. They had however a series of properties not described yet for PMN-ectosomes, i.e. they expressed phosphatidyl-serine in the outer leaflet of their membrane similarly to apoptotic bodies and to vesicles released by platelets, they acquired enzymes originating from azurophilic granules on their membrane, and they targeted endothelial cells as well as monocytic THP-1 cells.

Using electron-microscopy we have previously shown that the vesicles shed by PMNs had a size of approximately 50 to 200 nm[10]. Here, the vesicles were analysed by FACScan, which allowed us to further define them. A distinct population of membrane-particles was clearly identified and when compared to scattering-characteristics of synthetic beads (diameter 200 nm and bigger) these membrane-particles/vesicles had the same wide range of sizes as seen on the electron-microscopy images[10]. All the events registered in the defined FACScan window incorporated a lipid marker for membranes indicating that no artifacts such as aggregates of proteins were present. The intensity of labeling with this marker was directly correlated with the size of the vesicles confirming that the lipid-membrane increased together with the size of the vesicle as estimated from the scattering data. Interestingly all positive protein and lipid markers used showed the same increase in fluorescent intensity with the increase in size, suggesting that vesicles may differ in size but not in their general membrane composition. Thus activated PMNs shed one type of vesicles, which might however not have identical properties because of their size.

Ectosomes should contain cytosolic proteins. The presence of actin within and not on the surface of PMN-derived vesicles confirmed that they are released as intact rightside-out membrane-vesicles with a cytosolic content. Actin has been shown to be associated with vesicles released by mechanically-stressed fibroblasts, where it is thought to be involved in vesicle budding[13]. Actin also has been proposed to play a role in specific protein retention during *in vitro* budding of erythrocyte-vesicles[30]. It is likely that actin together with other cytoskeletal components plays a role in the formation of PMN-derived ectosomes as well. Stimulatory agents induce the shedding of membrane-vesicles mainly by inducing first an influx of calcium into the cell, which is followed by the scrambling of lipids between the two layers of the cell-membrane, and possibly by an active transport of phosphatidyl-serine[5, 31, 32]. These reactions result in loss of the physiological asymmetry of the membrane and the expression of phosphatidyl-serine on the outer leaflet. Once these events have taken place, vesicles are shed by budding from the cell-membrane. We expected therefore ectosomes to express phosphatidyl-serine and could demonstrate it by the binding of AnV. However, normal cells can restore this asymmetry over time[14], which was not the case for ectosomes (data not shown). This may reflect insufficient flipase-activity to restore the asymmetry owing to an insufficient amount of the enzyme and/or the absence of ADP/ATP-regeneration within ectosomes. Phosphatidyl-serine exposed on different types of micro-vesicles/ectosomes, including those derived from platelets, monocytes and endothelial cells, mediates procoagulant activities by recruiting coagulation factors[5, 20, 33-37]. Similar procoagulant properties have been attributed to PMN-derived ectosomes[38].

The second ectosomal protein identified by 2-DE/protein sequencing was annexin III. Annexin III is mainly expressed in PMNs. In resting PMNs it is associated with cytoplasmic granules, but translocates to the plasma membrane as soon as the cells get activated[39]. It is known to bind indiscriminately to membrane surface in a calcium dependent manner. Thus, it

is very likely that annexin III associates to forming ectosomes at the time of PMN-activation. The physiological role(s) of this protein are hitherto still largely unknown. However, annexin III has been proposed to mediate vesicle-fusion in secretory cells and might be involved in exocytosis. It might therefore play an active role in the process of ectocytosis as well.
We have previously shown by co-immunoprecipitation, that CR1-bearing ectosomes co-express HLA-I, CD46, CD55 and CD59, while CR1-bearing ectosomes did not co-immunoprecipitate with detectable amounts of CD14[10]. FACScan-based analysis confirmed these findings, adding evidence for a specific ectosomal protein expression, not simply corresponding to protein expression on the PMN cell-surface membrane (absence of CD14 but also of CD32 and CD87). The selection for ectosomal protein expression did not segregate between transmembrane- (TM-) and GPI-anchored proteins since both types of molecules were present (e.g. $CR1_{TM}$, $CD55_{GPI}$), respectively absent (e.g. $CD14_{TM}$, $CD32_{GPI}$) on ectosomes. However our technique did not allow us to define whether some of the molecule were enriched in the ectosomes as compared with the PMN-membrane. Such enrichment has been described on ectosomes released by erythrocytes for GPI-anchored proteins, but interestingly for CR1 as well[40]. Taken together these data would suggest that at the time of ectocytosis the shuffling of the membrane proteins follows rules, which are not specifically dependent on GPI anchoring.

Upon activation, PMNs are known to shed very efficiently L-selectin (CD62L), as well as CD11b[41-46], CR1[47] and many other molecules[48-50]. Interestingly, ectosomes were found to express some of these molecules. Thus, early during activation PMNs release a set of cell-surface molecules by proteolytic cleavage but also by ectocytosis, indicating that both processes take place in an orchestrated manner. Shedding of cell surface molecules is thought to be a means for a rapid adaptation of the cellular phenotype[49]. For instance shedding of L-selectin (CD62L) is known to be important during extravasation of PMNs in the transition

from a rolling phenotype to firm endothelial adhesion[51, 52]. In this respect, ectocytosis might be viewed as an additional efficient mechanism to modify the PMN cell-surface phenotype. On the other hand, the presence of CD62L on ectosomes might be essential for their adhesion properties. This observation suggests as well that enzymatic cleavage differs on the PMN cell-membrane and shed ectosomes. Smith et al. have shown recently that the susceptibility of erythrocytes to secretory phospholipase A2 after exposure to calcium ionophore was related to the specific properties of the lipid membrane, i.e. it was proportional to the increased order of membrane lipids[53]. One might speculate that, for ectosomes as well the enzymatic susceptibility of membrane molecules is related somehow to changes in the membrane lipid organisation.

Among the most fascinating characteristics of PMN-derived ectosomes was the fact that they selectively combined molecules well segregated in resting PMNs, i.e. a series of adhesion molecules and many but not all enzymes stored within azurophilic granules. This unique grouping was related to different processes – selection of membrane proteins for adhesion molecules and specific binding of enzymes to the outer leaflet of the ectosome membrane. How enzymes are translocated to this site and the types of binding are ill defined. Taking advantage of MPO-deficient PMNs we studied the mechanism of MPO translocation to ectosomes. Ectosomes released from MPO-deficient PMNs were found to acquire MPO when exposed to S/N of degranulated normal PMNs (containing soluble MPO) or to purified MPO. This binding is not unique for ectosomes, since MPO is known to bind to resting PMNs as well[25]. Since the ectosomal membrane is negatively charged electrostatic interactions with cationic MPO are very likely to contribute to the binding. In contrast, PR3, another cationic enzyme present on PMN-derived ectosomes, has recently been shown *not* to rebind to PMN-membranes[25]. PR3 instead translocates to the cell surface directly, exhibiting strong (possibly covalent) interactions with the membrane. Whether a fraction of MPO becomes

translocated in a similar manner cannot be excluded. Elastase, MPO and MMP-9 were in an enzymatically active form on whole, intact ectosomes. After disrupting the ectosomes by sonication there was no increase in the enzymatic activity of MMP-9 and elastase suggesting that this activity was limited to the outerside of the ectosome membranes with no storage compartment inside ectosomes.

In addition to being present on PMN-derived ectosomes, active MMP-9 has been demonstrated on ectosomes derived from fibrosarcoma, breast carcinoma cells as well as endothelial cells[22, 23, 54]. PMNs store MMP-9 in three different pro-forms: monomers of 92 kDa, dimers of 220 kDa and MMP-9/lipocalin heterodimers of about 130 kDa[55]. All forms are released upon PMN-activation and we found all storage-forms on the surface of ectosomes. Subsequent activation of MMP-9 is tightly controlled by ubiquitous tissue inhibitor of matrix-metalloproteinase-1 (TIMP-1)[56, 57]. Ectosomal MMP-9 might therefore become fully active thanks to elastase, which is known to inactivate TIMP-1[58]. Distinct ectosomal sets of molecules may locally shift the balance between inhibitory and activatory molecules, thereby allowing specific enzymes to escape inhibition. Besides acting on molecules in their microenvironment, ectosomal enzymes might also act on proteins expressed on the same ectosomes. This might explain the absence on ectosomes of certain proteins sensitive to proteolytic cleavage [e.g. CD14, uPAR (CD87)][59-61]. CD14 for instance has been shown to be sensitive to proteolytic cleavage by cathepsin G[59]. Cathepsin G is released at the time of PMN-activation; whether it is present on the surface of ectosomes is yet unknown. However, both soluble and ectosome-bound cathepsin G might cleave off CD14 from the surface of ectosomes.

The overall cleavage process would however be very selective, since other molecules such as L-selectin, although shed from PMNs, are not cleaved from the surface of ectosomes. Uniting

adhesion molecules with antimicrobial enzymes (MPO, PR3 and/or elastase)[62-64] or matrix-degrading enzymes (elastase, PR3 and/or MMP-9)[63, 65-68] may target “effector” molecules to specific locations. This might be beneficial, e.g. by focusing antimicrobial activity on opsonised bacteria, but also contribute to local tissue destruction or cell activation.

The expression on ectosomes of negatively charged lipids (such as phosphatidyl-serine) as well as different adhesion molecules suggested that they might be able to specifically bind to cells. Indeed ectosomes selectively and specifically bound HUVEC and THP-1 cells with high affinity/avidity, and not to erythrocytes. Many receptor(s) or molecule(s) of ectosomes may be involved in this binding, including L-selectin, and phosphatidyl-serine[69-72]. Interestingly, the addition of annexinV did not decrease or even block the binding of ectosomes to THP-1 cells (data not shown). We believe that the binding of ectosome to selective cell types might not occur through just one type of molecular interaction; blockade of one mechanism might be compensated by several others. Importantly, cytochalasin D did not prevent THP-1 cells to become fluorescent in our assays (data not shown), confirming that our experimental conditions tested binding. Longer incubation times might allow phagocytosis of ectosomes to occur. Whether phagocytosis of ectosomes induces a pro-inflammatory or anti-inflammatory state in monocytes remains an intriguing question, particularly when one considers that ectosomes may have some similarities with apoptotic bodies which are under physiological circumstances cleared without producing harm[73-76]. C1q has been shown to bind to the surface blebs of apoptotic cells, a binding which occurs via the globular heads of C1q[77]. Recent evidence suggests that the binding of C1q is important for the non-phlogistic clearance of apoptotic cells, thus preventing the development of autoimmunity against the many newly expressed antigens on apoptotic cells. Interestingly, C1q-deficiency individuals develop an autoimmune disease, which is very similar to systemic lupus erythematosus[78]. Here we showed that ectosomes bind C1q from serum similarly to

apoptotic cells. We have no evidence yet to suggest that the binding and clearance of ectosomes is regulated by C1q binding, but from the foregoing this possibility remains open. In summary, many of the biochemical characteristics of ectosomes of PMNs have been analysed in the present work. The observations made open new avenues in the way we think about the biological role of the released ectosomes. In addition, the clear definition of ectosomes, as detailed here, could serve as a basis for a unified terminology, which would replace, the multiple names used until now (microparticles, microvesicles, etc.). This would also underline the difference between ectosomes and exosomes which are formed by another process and might have very different functions.

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# Part II:

## Activated Polymorphonuclear Neutrophils disseminate Anti-inflammatory Microparticles by Ectocytosis

### Abstract

Upon activation, human neutrophils release microparticles, called ectosomes, directly from the cell surface membrane. Microparticles from platelets, endothelial cells and monocytes were reported to support coagulation or to modulate vascular homeostasis by activating monocytes as well as endothelial cells. We find that neutrophil ectosomes have no pro-

inflammatory activity on human macrophages as assessed by the release of IL-8 and TNFα. On the contrary, ectosomes increase the release of TGFβ1, suggesting that ectosomes downmodulate cellular activation in macrophages. PMN ectosomes are able to block macrophages' inflammatory response to zymosan and LPS, an effect that is partially mediated by the early-phase TGFβ1 secretion. Ectosome-cell contact was sufficient for their immunomodulatory function as shown by blocking phagocytosis with cytochalasin D. Thus, neutrophils release potent anti-inflammatory effectors, in the form of ectosomes, at the earliest stage of inflammation, already providing a drive to its resolution.

## Introduction

Polymorphonuclear neutrophils (PMN) constitute the bulk of the leukocytes found in our blood. PMN ingest and eventually kill invading pathogens by means of potent antimicrobial agents released during the process of degranulation. As this microbicidal weaponry largely lacks specificity, it can lead to severe tissue damage if not controlled or secluded adequately from surrounding tissue[1]. At the time of degranulation, activated PMN release small microvesicles directly from the cell surface membrane. Many eukaryotic cells, including tumors cells, release vesicles by ectocytosis (ectosomes), either spontaneously, or in response to various stimuli[2-14]. Data on the function(s) of ectosomes have accumulated recently[6,7,9,11,15-17]. So far, ectosomes have been associated with pro-coagulant and pro–inflammatory effects. Ectosomes derived from endothelial cells have been described to induce pro-coagulant activity in monocytic cells[18], whereas ectosomes from platelets and monocytes were shown to directly promote hemostasis and induce inflammation by activating endothelial cells[9,11]. MacKenzie et al. described monocyte-derived ectosomes as being important pro-inflammatory agents by mediating the rapid secretion of IL-1β[6].

We recently described characteristics and molecular properties of ectosomes released by human PMN[19,20]. Here we show that PMN derived ectosomes, unexpectedly, feature immunosuppressive/anti-inflammatory functions. Challenging a linear model of induction and resolution of inflammation, our data suggest a more complex balance of pro- and anti-inflammatory events initiated in a parallel manner.

## Material and Methods

*Antibodies and Reagents*

Zymosan A from *Saccharomyces cerevisiae* and cytochalasin D were from Sigma (St-Louis, MO) and LPS was from Calbiochem (La Jolla, CA). Human TGFβ1 Duoset® ELISA development system as well as neutralizing monoclonal mouse anti-human-TGFβ antibodies (αTGF-Abs) were from R&D Systems (Minneapolis, MN). OptEIA ELISA kits for IL-8, IL-10 and TNFα were from Becton Dickinson (San Diego, CA).

*Isolation and stimulation of PMN , collection of ectosomes*

PMN were isolated from fresh buffy coats according to the technique described previously[19]. Briefly, a fresh buffy coat was diluted 1/1 (v/v) with PBS-EDTA (2 mM), mixed gently with 0.25 vol of 4% Dextran T500 (Amersham Pharmacia Biotech, Dübendorf, Switzerland), and left for 30 minutes for erythrocyte sedimentation. The leukocyte-rich supernatant was aspirated and centrifuged for 10 minutes at 200*g*. The pellet was resuspended in 9 mL of ultrapure water to lyze erythrocytes. Isotonicity was restored by addition of 3 mL of KCl (0.6 M) and 40 mL of NaCl (0.15 M). Cells were then centrifuged 10 minutes at 350*g*, and resuspended in 20 mL of PBS-EDTA. This suspension was layered over 20 mL of Ficoll-Hypaque (Sigma, St-Louis, MO) and centrifuged for 30 minutes at 350*g*. The PMN-rich pellet was recovered and washed twice in PBS-EDTA. All manipulations were performed at 4°C, thus minimizing PMN-activation.

For stimulation, PMN ($10^7$ cells/mL) were diluted 1/1 (v/v) in pre-warmed (37°C) RPMI 1640 (Life Technologies, Basel, Switzerland) with 1 µM fMLP (final concentration; Sigma, St-Louis, MO), and incubated for 20 minutes at 37°C. PMN were removed by centrifugation (4000*g* at 4°C). The ectosomes contained in the supernatant were concentrated with Centriprep® centrifugal filter devices (10,000 MW cut-off, Millipore Corporation Bedford,

MA) and stored in aliquots at -80°C until use. The load of ectosomes was estimated in units (i.e. one unit of ectosomes corresponding to the ectosomes released by $10^7$ PMN).

*Isolation and culture of human monocyte derived macrophages (HMDM)*

Monocytes were isolated from fresh buffy coats as previously described[21]. Briefly, a buffy coat was diluted 1/1 (v/v) with HBSS, layered over Ficoll-Hypaque (Sigma, St-Louis, MO) and centrifuged for 30 minutes at 350*g*. Monocytes were recovered, washed twice in HBSS and layered over a Percoll gradient. Percoll was prepared mixing 1 volume NaCl 1.5 M with 9 volumes of Percoll (Sigma, St-Louis, MO). The Percoll gradient was done mixing 1.5:1 (v/v) isosmotic Percoll with PBS/Citrate ($NaH_2PO_4$ 1.49 mM; $Na_2HPO_4$ 9.15 mM; NaCl 139.97 mM; $C_6H_5Na_3O_7$ .$2H_2O$ 13mM; pH 7.2). Isolated monocytes were resuspended at 2 x $10^6$ cells/mL in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine (DMEM+). Monocytes were then allowed to adhere for one hour at 37°C on culture plates. Adherent monocytes were washed 3 times with prewarmed DMEM+ and finally incubated for 7 days in DMEM+ supplemented with 10% normal human serum (pooled from 40 healthy donors). The culture was maintained in 5% $CO_2$ at 37°C and the medium was changed at days 3 and 7. Macrophages were used between days 7 and 10.

*Activation of HMDM*

HMDM were washed several times with pre-warmed DMEM+ without serum. Subsequently, each well was filled with 200 μL (final volume) fresh DMEM+ without serum. Zymosan (5 μg/mL final concentration), LPS (10 ng/mL final concentration) and/or ectosomes were added and supernatants were collected 24 hours later (if not stated otherwise). For kinetic experiments, supernatants were collected at the time points 1, 2, 4, 8 and 24 hours. For some experiments, HMDM were pre-treated with 0.5 μM cytochalasin D in DMEM+ for 60 minutes at 37°C prior to washes and incubation with zymosan and/or ectosomes. For the dose-

dependent activity of ectosomes on activated HMDM, the highest dose of ectosomes used was equivalent to the amount released by $10^8$ PMN. For all other assays, the amount of ectosomes were set to obtain good but not complete inhibition of HMDM activation. Assays were performed in triplicate. Presented results are representative of three independent experiments. Data represent mean ± standard deviation.

*Collection of supernatants and analysis of cytokines*

HMDM supernatants were collected, put on ice and spun for 10 minutes at 2,000 rpm at 4°C (Mikro 24-48R centrifuge from Hettich, Bäch, Switzerland) to remove cellular debris. Finally, supernatants were collected and stored at –80°C until use. Cytokine concentrations were determined by ELISA according to manufacturers' instructions.

*Membrane labeling of ectosomes*

An amphiphilic cell linker dye kit (PKH67, Sigma, St-Louis, MO) was used, following the labeling procedure provided by the manufacturer. Briefly, ectosomes resuspended in 200 μL Diluent C were incubated with 200 μL diluent C/dye solution (dye diluted 1/200) for a minute at room temperature, with gentle shaking. 1 mL RPMI 1640 (without phenol red) was added to stop the reaction. Labeled ectosomes were separated from the remaining unbound dye by ultracentrifugation (45 minutes, 160,000*g*/4°C) and washed with 0.9% NaCl.

*Confocal fluorescence microscopy*

HMDM were generated as described above on 8-well culture slides (Falcon / Becton Dickinson, NJ, USA). After 7 to 10 days of culture, macrophages were washed several times with serum free DMEM+ medium and incubated with fluorescently labeled ectosomes (as described above). Analysis was performed on a Axiovert Confocal Laser Scanning Microscope (LSM 510) from Zeiss AG (Feldbach, Switzerland).

## Results

*PMN ectosomes do not induce pro-inflammatory activity in macrophages*

To investigate whether ectosomes derived from neutrophils have a similar pro-inflammatory effect as has been reported for ectosomes of different cellular origin, we co-incubated PMN ectosomes with human monocyte-derived macrophages (HMDM) for 24 hours. Supernatants were analyzed for IL-8, TNFα, IL-10 and TGFβ1. No stimulating or activating effect of ectosomes on macrophages was observed (Fig 1A), whereas zymosan and LPS induced the release of IL-8, TNFα and IL-10. Surprisingly, ectosomes increased the release of the anti-inflammatory cytokine TGFβ1.

*PMN ectosomes downregulate the inflammatory response of HMDM to zymosan*

Through co-incubation of zymosan or LPS with ectosomes we assessed whether ectosomes, besides being intrinsically non-inflammatory, alter the response of HMDM to pro-inflammatory stimuli. Zymosan and LPS induced the release of IL-8, IL-10 and TNFα, but not of TGFβ1. In presence of zymosan, ectosomes significantly downregulated the release of IL-8 and IL-10, completely blocked the secretion of TNFα but increased the release of TGFβ1. Ectosomes were best at counteracting the pro-inflammatory response to zymosan, although with regards to TNFα and TGFβ1, the response to ectosomes was very similar. Interestingly, the overall effect of ectosomes on activated macrophages could not be blocked by pre-incubating ectosomes in normal human serum (i.e. in the presence of serum proteins) for 30 minutes at 37˚C. Furthermore, as shown in figure 1C, ectosome-induced downregulation of IL-8, IL-10, TNFα secretion and induction of the secretion of TGFβ1 was dose-dependent. In the highest amount used in our assay, ectosomes completely blocked the inflammatory response of HMDM. Noteworthy, the release of cytokines by macrophages and its inhibition/promotion by ectosomes after activation with stimuli has an intrinsic variability due to nearly each experiment being carried out with cells and ectosomes isolated from

different blood donors. This variability is particularly reflected in figure 1A, B and C by the different background releases of TGFβ1 in resting macrophages.

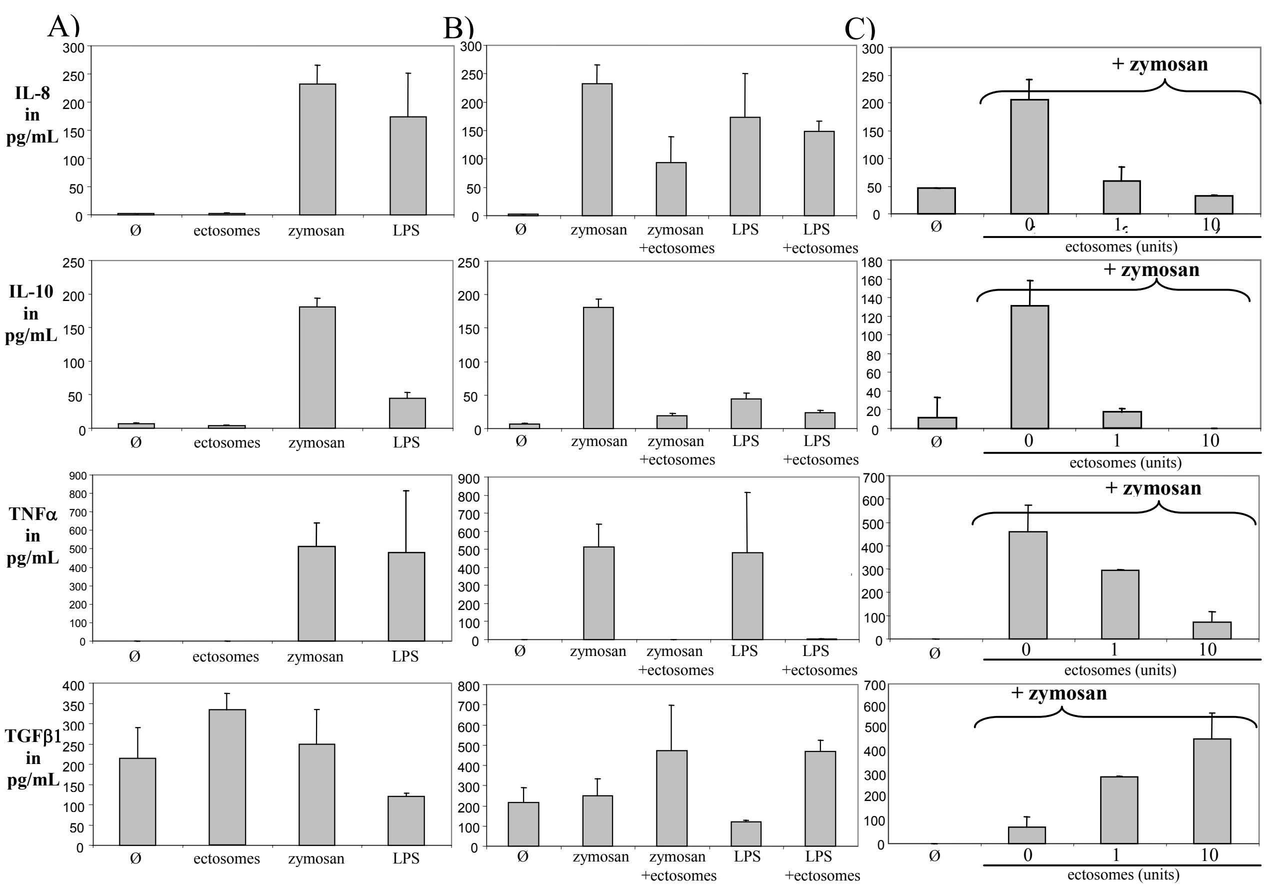


**Figure 1: PMN ectosomes are not pro- but anti-inflammatory**

A) HMDM were incubated with medium alone (Ø), with medium supplemented with ectosomes (1 unit), zymosan or LPS for 24 hours. Supernatants were analyzed for IL-8, IL-10, TNFα and TGFβ1. B) HMDM were incubated with medium alone (Ø), with medium supplemented with zymosan or LPS, with or without ectosomes for 24 hours. Supernatants were analyzed for IL-8, IL-10, TNFα and TGFβ1. C) HMDM were incubated with medium alone (Ø), with medium supplemented with zymosan with (1 or 10 units) or without ectosomes for 24 hours. Supernatants were analyzed for IL-8, IL-10, TNFα and TGFβ1.

*Kinetic response of HMDM to ectosomes and/or zymosan*

Next, we assessed whether the diminished release of pro-inflammatory cytokines in response to ectosomes was due to a true blockade of intra-cellular pro-inflammatory pathways or rather a delayed response. We therefore investigated the time dependent effect of ectosomes on the cytokine secretion of both, resting and zymosan-stimulated HMDM (Fig. 2). Ectosomes induced a rapid and sustained release of TGFβ1 in resting and stimulated HMDM. In the presence of zymosan, ectosomes had a profound anti-inflammatory activity on HMDM detectable at all time points. The kinetics of IL-8, IL-10 and TNFα release by activated macrophages were similar in the absence and presence of ectosomes, although in the latter the amplitudes were significantly decreased. This indicates that the immunosuppressive activity of ectosomes is not due to a delay in the response of macrophages to pro-inflammatory stimuli, but rather to an active suppression of cell activation.

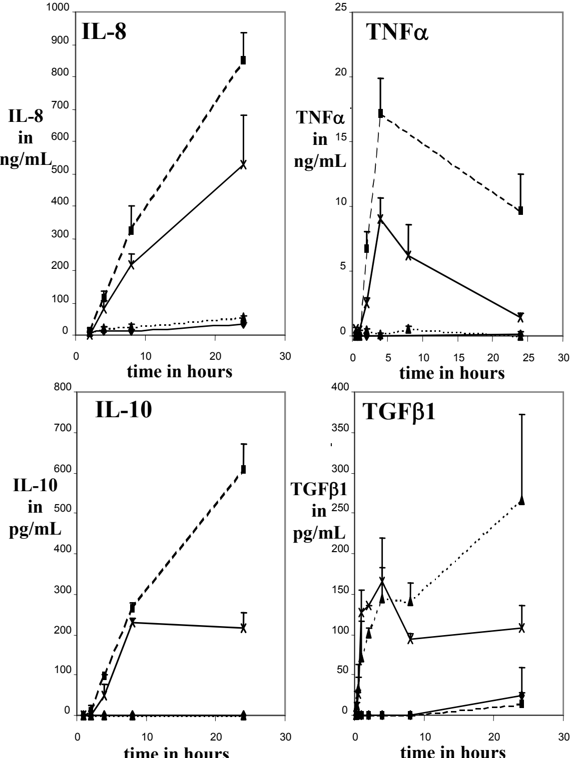


**Figure 2: Kinetic of cytokine release by HMDM dependent on zymosan and/or ectosomes**

HMDM were incubated with medium alone (Ø) with or without ectosomes (1 unit) and with medium supplemented with zymosan with or without ectosomes for up to 24 hours. Supernatants were harvested after 1, 2, 4, 8 and 24 hours and analyzed for IL-8, IL-10, TNFα and TGFβ1. Legend: —◆— HMDM alone; - -■- - HMDM + zymosan; ···▲··· HMDM + ectosomes; —×— HMDM + ectosomes + zymosan.

*Ectosomes are phagocytozed by HMDM*

We previously observed that ectosomes bind specifically to macrophage-like cells differentiated from THP-1 cells [20]. Here, we addressed the question whether ectosomes are phagocytozed by macrophages and whether the process of phagocytosis is involved in the immunosuppressive function of ectosome on macrophages.

We performed confocal fluorescence microscopy of HMDM after incubation with fluorescently labeled ectosomes. As shown, ectosomes do not only bind to macrophages but are internalised as well (Fig. 3A). We performed assays with HMDM that were kept in medium alone and others that were pre-incubated with cytochalasin D (cytD), a potent inhibitor of phagocytosis. In absence of cytD, the vast majority of macrophages have phagocytozed ectosomes after 30 minutes, as reflected by the intense fluorescent staining of most of the cells (i). In contrast, cytD pre-incubated macrophages show a marked decrease in fluorescence and hence uptake of ectosomes (Panel iii). At higher magnification, cells that have ingested ectosomes accumulated fluorescence in intracellular compartments (Panel ii), which were not visible after pre-treatment with cytD (Panel iv).

We then used again cytD to determine whether phagocytosis is required for ectosomes to fulfil their anti-inflammatory effects on macrophages. Since CytD significantly influenced the TNF$\alpha$ response of HMDM in the presence or absence of zymosan, we excluded TNF$\alpha$ results from our analysis. As shown in the panels B-C (Fig. 3), inhibiting ectosome-phagocytosis could not block the anti-inflammatory effect of ectosomes on HMDM.

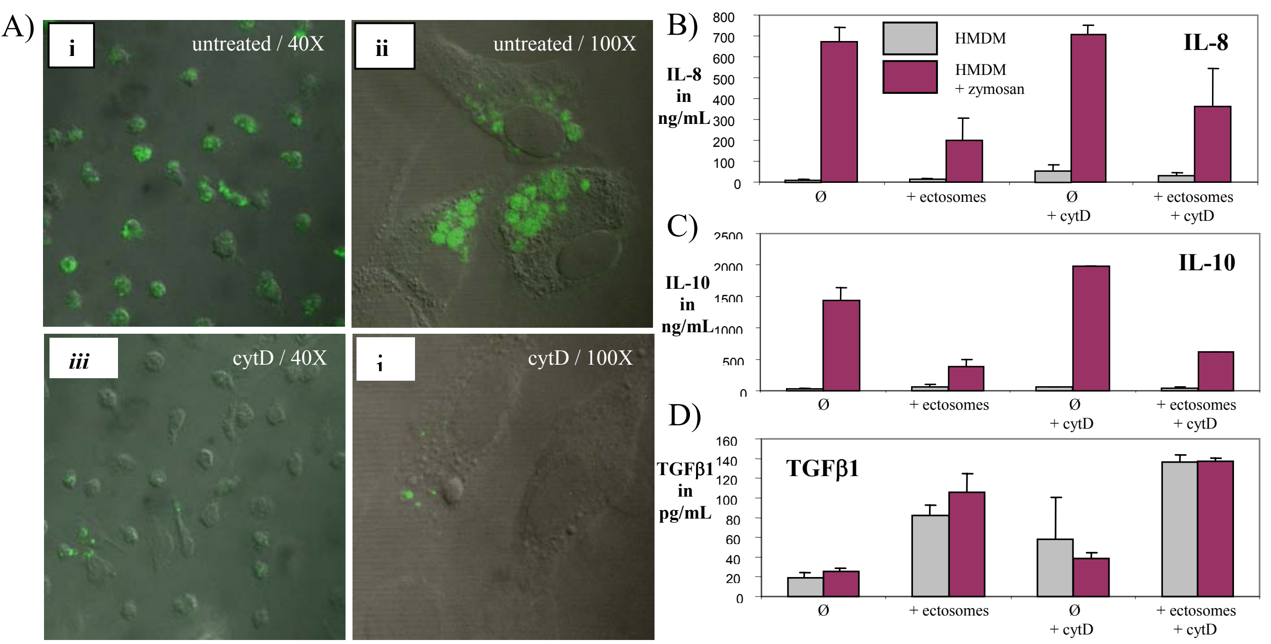


**Figure 3: Confocal microscopy of phagocytosis and phagocytosis independent anti-inflammatory activity of ectosomes**

A) HMDM were incubated with fluorescently labeled ectosomes for 30 minutes, fixed and analyzed by confocal laser microscopy (i-ii). Alternatively, macrophages were pre-incubated cytochalasin D (cytD) prior to the addition of ectosomes (iii-iv). B)-D) HMDM were incubated with medium alone or with medium supplemented with zymosan for 24 hours. Alternatively, ectosomes (1 unit) were added to the incubation medium with or without pre-incubating the macrophages with cytD.

*Effect of neutralizing anti-TGFβ antibodies on anti-inflammatory activity of ectosomes*

In order to understand how ectosomes exert their immunosuppressive activity on macrophages. The kinetic data presented earlier suggested that the first event occurring during co-incubation of macrophages with zymosan and ectosomes is the ectosome-induced release of TGFβ1. We tested whether the rapid rise in local TGFβ1 concentration as shown in Fig. 2 is responsible for the subsequent downregulation of IL-8, IL-10 and/or TNFα release in activated macrophages. As shown in Fig. 4, neutralizing αTGF-Abs had a lowering effect on the anti-inflammatory activity of ectosomes. This effect ranged from complete blockade of

ectosome-function with respect to downregulation of IL-8, to only partial inhibition of the downregulatory activity of ectosomes in the case of IL-10 and TNFα.

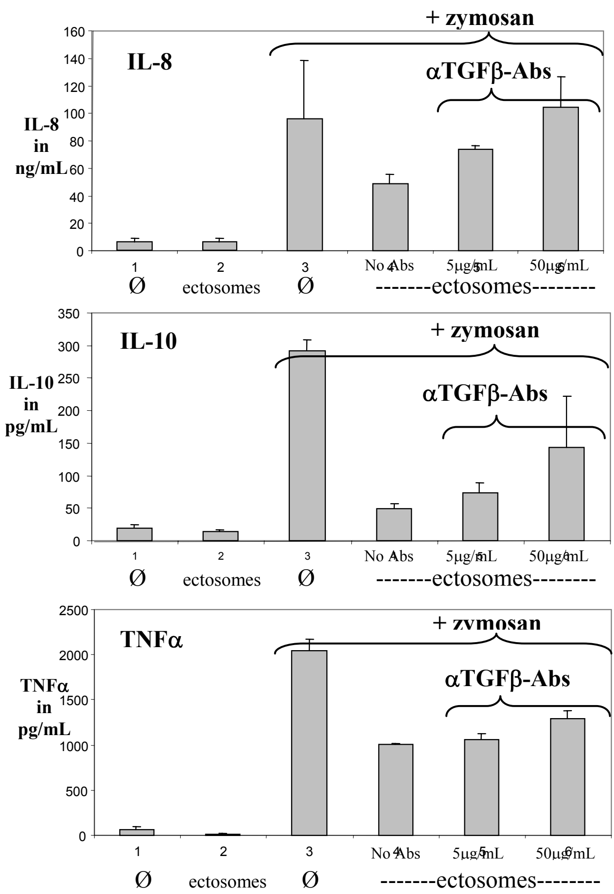


**Figure 4: Effect of neutralizing anti-human-TGFβ antibodies on anti-inflammatory activity of ectosomes**

HMDM were incubated with medium alone (Ø), medium with ectosomes (1 unit) or with medium supplemented with zymosan for 24 hours. Alternatively, zymosan-medium was supplemented with ectosomes alone (no antibodies) or ectosomes with 5mg/mL or 50mg/mL of αTGFβ-Abs. Supernatants were analyzed for IL-8, IL-10 and TNFα.

## Discussion

This study associates ectosomes to an active role in the resolution of inflammation. This function of PMN derived ectosomes is striking, as it counterbalances the immediate pro-inflammatory effects of many of the substances released by activated PMN. Our data challenge the sequential model of induction and resolution of inflammation, and suggest a balanced occurrence of pro- and anti-inflammatory events, orchestrated in a parallel manner.

Ectosomes share with apoptotic bodies the expression of phosphatidylserine (PS) in the outer membrane leaflet. Similarly to apoptotic cells, the uptake of ectosomes by macrophages might depend on the exposure of PS in the outer membrane leaflet[22]. In addition, and in analogy to apoptotic cells, the uptake of ectosomes by macrophages is accompanied by a reprogramming of the macrophages, featuring an anti-inflammatory phenotype, and this biological effect does not require phagocytosis.

The receptor for PS has recently been shown to be directly involved in the resolution of inflammation by inducing the release of TGFβ1 in macrophages that have ingested apoptotic cells[23,24]. Here, we show that ectosomes, in further analogy to apoptotic bodies, promote the dose-dependent release of TGFβ1 in activated as well as resting macrophages, and an inhibition of IL-8, IL-10 and TNFα secretion in activated macrophages. Anti-TGFβ-Abs counteracted the inhibition of IL-8 release induced by ectosomes suggesting that IL-8 release is regulated by autocrine and/or paracrine action of TGFβ1; similar observation has been made with apoptotic cells. However, and in distinction to what happened with apoptotic cells, αTGF-Abs had little effect on the inhibition of IL-10 and TNFα release[25].

It thus seems likely that ectosomes and apoptotic cells engage distinct but overlapping but different pools of receptors on the surface of macrophages.

Our report follows a series of papers on functions of ectosomes, alternatively called microparticles or microvesicles[6,7,9,11,15,16]. In most cases, ectosomes were described as having pro-inflammatory properties. Plasma counts for microparticles of various cellular origin were analyzed in a number of pathophysiological situations and such microparticles were found to induce endothelial dysfunction[8,14,26]. This contrasts our data for PMN, suggesting that the cellular origin of ectosomes as well as the target cells may determine their biological response. This fact is illustrated by recent work of Mesri *et al*. that described activating properties of PMN microparticles on endothelial cells[15,27]. However, the microparticles used in these experiments were isolated after several hours. Microparticles were not selected for early-released ectosomes, as used in this work, and may have contained early apoptotic components and microparticle-membranes modified by hydrolysis, conferring to them very different properties[28].

In conclusion, ectocytosis of PMN early during activation might produce microparticles with immunosuppressive function and create an immediate counterweight for simultaneously unfolding pro-inflammatory mechanisms. Fascinating is that PMN, best known for their powerful and immediate phlogistic properties, release particles/ectosomes which seem to downregulate the inflammatory activity of the second wave of cells (macrophages) that will be attracted to the site of initial damage.

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# Part III:

## Microparticles released by Human Neutrophils adhere to Erythrocytes in the presence of Complement

### Abstract

The release of cell surface-derived microparticles, or ectosomes, has now been described for many different cell types. We reported previously on the binding of the first component of the classical pathway of complement, C1q, to the surface of ectosomes released by human neutrophils. Here, we show that the binding of C1q induces the activation and deposition of complement on the surface of ectosomes through the classical pathway. Furthermore, the opsonization of ectosomes by complement mediated their immune adherence to erythrocytes through complement receptor 1. Taken together, our data suggest an important role for complement and erythrocytes in the sequestration, and possibly clearance, of blood-borne microparticles stemming from PMN but potentially from other blood cells as well.

## Introduction

Whilst studying polymorphonuclear leukocytes (PMN), Stein and Luzio observed the release of vesicles that pinched off directly from the cell membrane when PMN were challenged with sublytic amounts of complement by a mechanism they termed ectocytosis[1]. A similar phenomenon occurs in many different cell types in response to a variety of stimuli. Ectocytosis occurs immediately after stimulation and is therefore timely and mechanistically well distinct from apoptosis. Beside PMN, endothelial cells, monocytes, platelets and erythrocytes are known to release microparticles by ectocytosis *in vitro* and *in vivo*[2-6]. For clarity, we will refer to "ectosomes" when describing such microparticles. A number of different functions have been attributed to ectosomes. They allow the rapid shedding of IL-1β, released by activated THP-1 cells[7]. Ectosomes can serve as intercellular protein-carriers for tissue factor and the chemokine receptor CCR5[8,9]. As far as platelet ectosomes are concerned, their most prominent feature is a pronounced procoagulant activity.

Microparticles of diverse cellular origin -probably mainly ectosomes- are present in blood of normal individuals[10]. Quantitative and qualitative changes, i.e. increased numbers of microparticles derived from different origins, were described in many diseases including sepsis[11], severe trauma[12], paroxysmal nocturnal hemoglobinuria[13], diabetes[14,15], acute coronary syndromes[16] and lupus[2]. Whether ectosomes of PMN are deleterious is unknown, but the level of blood microparticles of PMN correlated with the severity of sepsis[11]. However, even in sepsis the real number of these microparticles is surprisingly low, when compared to the high numbers released by activated PMN *in vitro*.

Here we present evidence that most ectosomes of PMN might not be free in circulation, as assumed until now. Indeed, ectosomes released by human PMN activate the classical pathway

of complement and fix C4 and C3 fragments. These opsonized ectosomes bind in turn to erythrocytes via the complement receptor 1 (CD35/CR1).

## Material and Methods

*Antibodies and Reagents*

Anti-human-C3d and -C4d antibodies (Abs) were from Quidel (Santa Clara, CA) and human C1q was from Calbiochen (San Diego, CA). Phycoerythrin coupled anti-glycophorin A and goat anti-mouse-Ig Abs were from Pharmingen (San Diego, CA) and Southern Biotechnology Associates Inc. (Birmingham, AL), respectively. Lepirudin (Refludan®) was from Hoechst (Zürich, Switzerland).

*Isolation and stimulation of PMN*

PMN were isolated from buffy coats as described previously[17]. PMN were resuspended in RPMI 1640 with 1 μM fMLP and incubated for 20 minutes at 37°C. PMN were removed by centrifugation. The supernatant was concentrated with centrifugal filter devices (Millipore, Bedford, MA) and stored in aliquots at -80°C until use.

*Membrane labeling of ectosomes*

An amphiphilic dye kit (PKH67, Sigma, St-Louis, MO) was used, following the labeling procedure provided by the manufacturer. Labeled ectosomes were separated from the remaining unbound dye by ultracentrifugation and washed with 0.9% NaCl.

*Complement activation assays on ectosomes by Fluorescence Activated Cell Scanning (FACScan)*

Ectosomes were isolated from concentrated PMN supernatant by ultracentrifugation. Ectosomes were resuspended in complement fixation diluent (CFD) supplemented with 10% heat-inactivated normal human serum (HI-NHS), normal human serum (NHS), C1q-deficient or C2-deficient serum and incubated for 30 minutes at 37°C. Ectosomes were then washed

with CFD and incubated with primary Abs. Detection was performed with goat anti-mouse-Ig-PE Abs.

*Fluorescence microscopy and FACScan of erythrocytes / whole blood*

Ectosomes were fluorescently labeled as mentioned above. Fresh erythrocytes were isolated from lepirudin anticoagulated blood, washed several times with RPMI 1640 and incubated with ectosomes in medium supplemented with 10% autologous lepirudin plasma for 30 minutes at 37ºC. Erythrocytes were washed gently several times with PBS and analyzed. Alternatively, reconstituted whole blood was used. The reconstitution of whole blood was performed isolating all blood cells from EDTA anticoagulated blood and adding autologous serum to regain initial blood volume.

## Results and discussion

Ectosomes bind C1q, the first component of the complement system[17]. We investigated whether this binding was followed by complement activation and deposition on the surface of ectosomes. As shown in Figure 1A panel (a), both C4 and C3 fragments were fixed onto ectosomes after exposure to 10% normal human serum. This complement activation was driven by the classical pathway as shown in a C2 deficient serum, in which C4 fragment binding took place, but which was not followed by C3 fragment fixation (panel (b)). This result indicated as well, that the alternative pathway was not capable by itself to opsonize the ectosomes with C3. In C1q deficient serum there was no complement fixation (panel (c)). Since this C1q deficient serum has been shown to have a functional mannan-binding pathway (kindly measured by J. Jensenius), we concluded that the initial deposition C1q was an absolute requirement for further complement activation. Finally, the classical pathway deposition of C3 was followed by membrane attack complex-formation on the surface of ectosomes (panel (d)).

Next, we tested whether complement deposited on ectosomes acts as an opsonizing agent, ultimately targeting ectosomes to erythrocytes, known to bear clusters of CR1 and function as carrier for complement-opsonized viruses, bacteria and immune complexes[18-20]. We performed FACScan experiments using fluorescent ectosomes and purified human erythrocytes, which were incubated in either lepirudin plasma (LP), LP-EDTA, C1q- or C2-deficient serum. We then assessed the fraction of erythrocytes having bound ectosomes. As shown in Figure 1B, non-opsonized ectosomes had only a small binding capacity to erythrocytes, whereas the number of erythrocytes bearing ectosomes increased almost 4-fold in the presence of 10% autologous LP. When EDTA was added to the plasma or when C1q-, or C2-deficient serum were used instead, the binding efficiency of ectosomes was decreased back to non-specific levels. C1q alone was unable to mediate binding even in supra-

physiological concentrations (200µg/mL). These results indicate that the immune adherence of ectosomes is complement dependent, requires C3 fragment binding, and that C1q in plasma, which can directly bind to CR1[21], does not significantly support this binding. In this respect ectosomes appear to have similar properties to immune complexes[22].

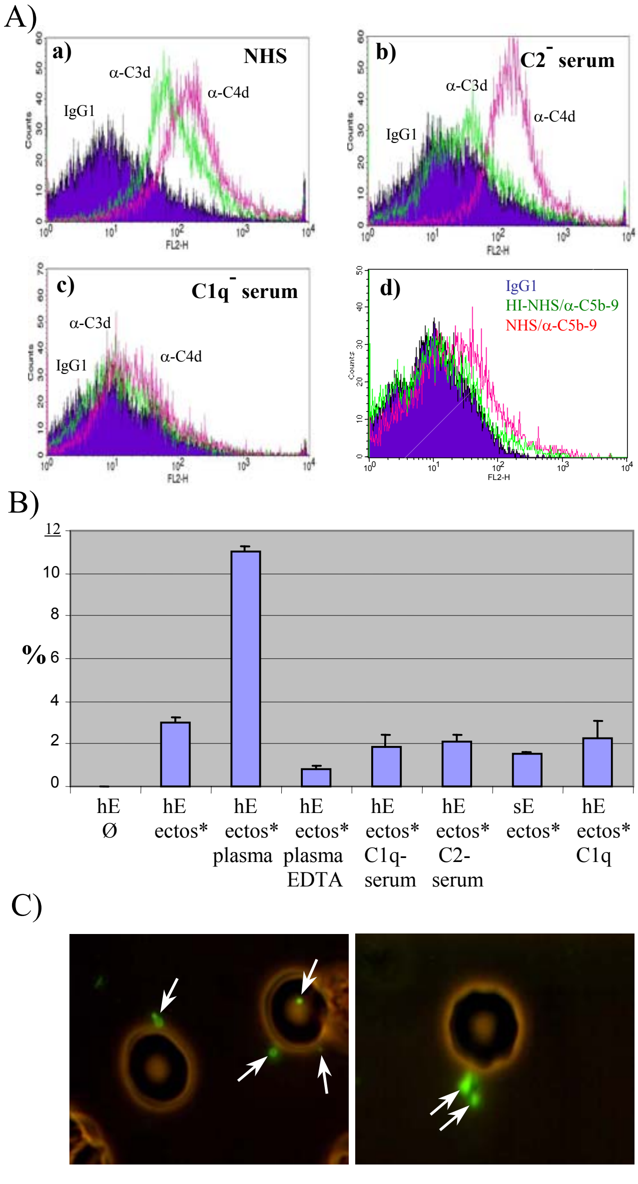


**Figure 1: Activation and deposition of complement on the surface of ectosomes / complement dependence and fluorescence microscopy of the binding of ectosomes to purified erythrocytes.**

A) FACScan histogram plots showing the deposition of C3- and C4-fragments and C5b-9 complexes on ectosomes. a)-c): the violet, green and red curves represent the fluorescences of ectosomes incubated with isotype control mouse(m)-IgG1 Abs, monoclonal m-anti-C3d Abs and monoclonal m-anti-C4d Abs, respectively, detected with goat anti-m-Ig-PE Abs. The experiments were performed in CFD supplemented with 10% NHS, C2- and C1q-deficient serum, respectively. Panel d) represents the deposition of MAC on ectosomes after incubation in NHS (red curve) or HI-NHS (green). B) Percentage of purified human (hE) or sheep (sE) erythrocytes that bound fluorescently labeled ectosomes (ectos*) in the presence of 10% autologous lepirudin plasma, plasma supplemented with EDTA, C1q- or C2-deficient serum or buffer supplemented with C1q. C) Erythrocytes were incubated with fluorescent ectosomes in the presence of 10% plasma, washed and analyzed by fluorescence microscopy.

Finally, the immune adherence of ectosomes was CR1 dependent, since it was completely abrogated by monoclonal anti-CR1 Abs (3D9), known to interfere with the C3b binding site on CR1, and absent on sheep erythrocytes, which lack CR1 (Fig. 1B). The same data were obtained by direct microscopy. The fine fluorescent ectosomes were seen attached on the surface of erythrocytes only in the presence of an intact classical pathway and normal human erythrocytes as illustrated in Figure 1C.

We then investigated the immune adherence of ectosomes in more physiological conditions, using fluorescent ectosomes and reconstituted whole blood. Anti-gpA Abs (αgpA-PE) were used to label erythrocytes. As shown in Figure 2, we could detect significant amounts of double positive events after incubation of reconstituted blood with ectosomes (6% of erythrocytes; > 90% of ectosome-positive events) (panel (c)).

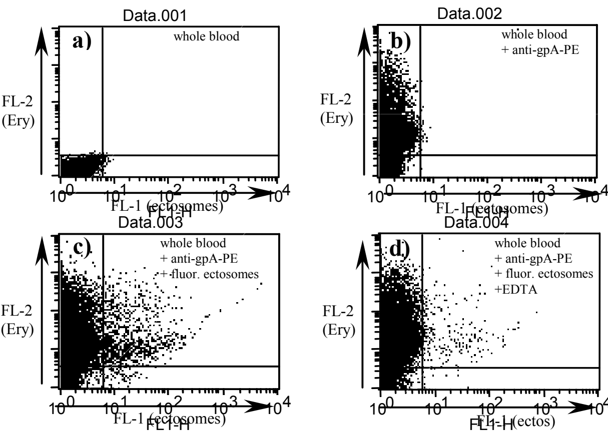


**Figure 2: Binding of ectosomes to erythrocytes in whole blood.**

FACScan dot plots showing the binding of ectosomes to αgpA-PE labeled erythrocytes (Ery) in reconstituted whole blood. a) Control fluorescence of whole blood in the absence of ectosomes. b) αgpA-PE labeled whole blood in the absence of ectosomes. c) αgpA-PE labeled whole blood incubated with fluorescent ectosomes. d) αgpA-PE labeled whole blood incubated with fluorescent ectosomes in the presence of EDTA.

We performed similar experiments adding EDTA to the reconstituted blood. As shown in panel (d), the counts of erythrocytes having bound ectosomes were reduced to 1.5%. Noteworthy, leukocytes did not bind significant amounts of ectosomes, probably because under physiological conditions the clustered distribution of CR1 on erythrocytes provided a significant binding advantage[23]. Taken together, these data suggest that the complement dependent immune adherence of ectosomes is likely to occur *in vivo*, i.e. in circulation, as well.

In conclusion, we suggest that the complement dependent immune adherence of ectosomes is a mechanism by which ectosomes are secluded in the circulation and possibly cleared subsequently in liver and spleen similarly to immune complexes. Although we tested only PMN-derived ectosomes for complement activation and immune adherence, other blood-borne microparticles might have similar complement activating properties. If this is the case, then erythrocytes may be considered as a buffer adsorbing microparticles, thus removing them from possible interactions with endothelial cells. The reduced number of CR1 on erythrocytes in SLE and other conditions might be detrimental for the physiological sequestration of microparticles. Finally the immune adherence of ectosomes should be taken into account when analyzing and interpreting quantitative and qualitative microparticle-counts in disease.

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**General Conclusion / Future perspectives**

Several conclusions can be drawn from the work presented here. First, human polymorphonuclear neutrophils (PMN)- derived ectosomes are released by a mechanism that selectively sorts cell-derived proteins into ectosome-membranes. This process, that remains to be fully unravelled, fits ectosomes with adhesion molecules and receptors, some of which might play a role in their binding affinities to phagocytic and endothelial cells, and active enzymes, including elastase, which might play a role in hemostasis. Second, PMN-ectosomes feature a marked anti-inflammatory activity when binding to human macrophages, a likely situation in inflamed tissue. It is tempting to assume that this function is mediated by phosphatidylserine, a negatively charged phospholipid confined to the inner membrane bilayer in healthy resting cells and presented in the outer layer on ectosomes; the reason being that this feature was associated to similar immunomodulatory activites observed for apoptotic cells[1,2]. Third and last, in contrast to the situation in tissue, the behaviour of ectosomes in blood was suggesting a somewhat different fate. Blood-borne ectosomes activate and bind complement, and are subsequently bound to erythrocytes, likely to mediate their clearance from the circulation, similarly to immune complexes.

The mechanism of ectocytosis is far from being understood. One possible means to release membrane vesicles is the loss of membrane lipid asymmetry[3-7]. Membrane phospholipid asymmetry is controlled by several proteins or protein-complexes: an ATP-dependent aminophospholipid-specific translocase, an ATP-dependent nonspecific lipid floppase and a $Ca^{2+}$-dependent non-specific lipid scramblase[8]. As the initial trigger, an increase in cytoplasmic $Ca^{2+}$-concentrations positively and negatively controls scramblase- and translocase-activities, respectively, and thereby promotes the loss of membrane asymmetry. In addition, $Ca^{2+}$-induced calpain activation favours vesicle formation and release of right-side oriented, phosphatidylserine exposing microparticles, corresponding to ectosomes[9,10]. The

collapse of membrane asymmetry is also facilitated by complement. Complement-induced release of microparticles exposing phosphatidylserine (PS) at their surface has been observed in endothelial cells, platelets and, as the first report describing and defining ectocytosis, in PMN[11-13].

It is unlikely that the breakdown of membrane lipid asymmetry is sufficient to induce ectocytosis. Many reports have suggested that structural proteins might be involved in the maintenance of lipid asymmetry and may thus modulate vesiculation[14,15]. The involvement of the cytoskeleton in ectocytosis is further suggested by the presence of filamentous actin in PMN-ectosomes, as shown in Part I. Recent data from Frasch *et al*. provide evidence for the selective enrichment of the phospholipid scramblase in detergent insoluble membrane regions (rafts) on the surface of fMLP-stimulated PMN[16]. Considering that rafts have been involved in the formation and the protein-sorting mechanism of exosomes it is conceivable that ectosomes could form preferentially at sites where specific membrane structure (rafts) colocalise with protein-complexes that mediate the collapse of membrane asymmetry.

The understanding of the molecular mechanisms of ectocytosis would be of great interest to functionally compare ectosomes from different cellular origin. If the PS-expression on the surface of ectosomes is a common feature of ectosomes regardless of the cell-type they derived from, one would expect to observe overlapping functions. Indeed, as alluded to above, the anti-inflammatory activity of PMN-ectosomes described in Part II is likely to be dependent on PS. The function of ectosomes would then result from common, mainly structural, elements conferring them “basic” characteristics and cell-specific protein-patterns adding individual functional traits. The function of ectosomes might also be influenced by the site of their genesis. Indeed, ectosomes released from circulating or extravasated PMN might encounter a different environment. Whereas blood-borne PMN-ectosomes are enabled to

interact with massive amounts of serum proteins, including complement and components of the coagulation cascade, ectosomes released from extravasated PMN face a different situation in the extracellular space in tissue. Common features, like the expression of PS, might then adopt several functions. PS, as a negatively charged phospholipid, is known to be essential for hemostasis by acting as a docking site for coagulation components mediating thrombin formation[17-20]. PMN-ectosomes might therefore promote coagulation in the circulation, as has been described for platelet-ectosomes[21]. Active human neutrophil elastase present on the suface of PMN-ectosomes could play an additional procoagulant role in inactivating tissue factor inhibitor (O. Gasser, unpublished results, collaboration with Prof. Engelmann, Munich)[22-24].

It would be interesting to confirm and describe the procoagulant activity of PMN-ectosomes in circulation and to investigate whether PMN-ectosomes retain at the same time their anti-inflammatory activity when released in blood, to rule out possible mutually exclusive mechanisms. Conceivably, in contrast to dendritic cell-derived exosomes efficiently used as immunogenic entities in vaccination experiments, blood-borne PMN-ectosomes might act as "anti-adjuvant", that is to say circulating immunomodulatory microparticles one could use to suppress or dampen the immune system[25-28]. Preliminary experiments (O. Gasser, unpublished results) using dendritic cells seem to justify the contention that ectosome-functions are not restricted to monocyte/macrophages and thus that ectosomes might affect the immune status long after their release during acute inflammation.

The effect of PMN-ectosomes on dendritic cells has to be confirmed and investigation on the molecular mechanisms ectosomes use to modulate the biology of macrophages and other immune cells might more precisely define the functional similarities and discrepancies between ectosomes and apoptotic cells. Whether PMN-ectosomes carry out more functions is an open question. The multitude of proteins expressed on their surface, with certainly more to

be identified, support this hypothesis. One might speculate that the enzymes present on the surface of ectosomes might carry out various functions: elastase might play a role in hemostasis, matrix metalloproteinase-9 in extracellular matrix remodelling and myeloperoxidase in antimicrobial defence. The multitude of possible functions of PMN-ectosomes is further extended by the various binding sites that might be targeted by the receptors/ligands expressed on their surface. Complete proteomic- and exhaustive binding-analyses as well as *in vivo* models will be necessary to gain more insight into ectosome's very interesting biology.

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# CURRICULUM VITAE

**Name:** **Olivier Gasser**
Position: PhD Student
Address: Immunonephrology Laboratory 414
Department of Research, ZLF
Kantonsspital Basel
Hebelstrasse, 20
CH-4031 Basel
Switzerland
Tel.: 061 265 38 91
Fax: 061 265 23 50
E-mail: Olivier.Gasser@unibas.ch

Date of birth: 9th of January 1976
Place of birth: Basel, Switzerland
Marital status: married
Private Addr.: 39, rue de Bourgfelden
68220 Hégenheim
France

## STUDIES

**Graduation** **Saint-Louis (F), 1994**
French Baccalauréat Section C „Mathematics and Physical Sciences"

**Biology Studies** **Basel (CH), 1994-1996**
Swiss Vordiplom in Biology II (Molecular Biology)

**Biotechnology Diploma** **Strasbourg (F), 1996-1999**
**Melbourne (AUS), jan-sep 1999**
Studies at the "Ecole Supérieure de Biotechnologie de Strasbourg" (ESBS) located at Strasbourg, France, including training courses at the Universities of Karlsruhe (Germany), Freiburg (Germany) and Basel (Switzerland).
Diploma Work done at the Walter and Elisa Hall Institute of Medical Research (WEHI) in Melbourne, Victoria, Australia, under the supervision of Dr. Andrew Lew.

**PhD** **Basel (CH), 1999-2004**
Work on the "characteristics, properties and function(s) of ectosomes released by human polymorphonuclear neutrophils" carried out in the Immunonephrology Laboratory within the Department of Research of the Kantonsspital Basel, under the supervision of Prof. Jürg A. Schifferli.

## SCIENTIFIC WORK

**Cyanobacteria Laboratory at the ESBS** **Strasbourg (F), 1997**
- Comparision of the glycolipidic composition of heterocyst membranes from different *Anabaena* cyanobacteria strains.

- Purification and isolation of *Anabaena* specific polyclonal antibodies.

Under the supervision of Dr. Cheng-Cai Zhang.

**Dynamics of G-protein-coupled receptors Lab (ESBS) Strasbourg (F), 1998**

- Construction of tachykinin receptor chimeras.
- Studies on the oligomerisation of chimeric tachykinin receptors using fluorescence resonance energy transfer (FRET).

Under the supervision of Dr. Jean-Luc Galzi.

**Autoimmunity and Transplantation Unit at WEHI Melbourne (AUS), 1999**

- Investigation on the increased immunity in mice through antigen-targeting to homing receptor mucosal addressin cell adhesion molecule 1 (MAdCAM-1).
- Generation of triple transgenic mice as a tool for assessing graft rejection in mice using luciferase and EGFP expression.

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