IMMUNOLOGICAL ASPECTS

Mycobacterium tuberculosis-induced neutrophil ectosomes decrease macrophage activation

Tonya Azevedo Duarte [a,b,*], Alberto Augusto Noronha-Dutra [c,d], Joilda Silva Nery [d],
Samantha Brum Ribeiro [b], Thassila Nogueira Pitanga [d], José R. Lapa e Silva [b], Sérgio Arruda [d],
Neio Boéchat [b]

[a] Health Science Institute, Federal University of Bahia, Salvador, BA, Brazil
[b] Multidisciplinary Research Laboratory, Clementino Fraga Filho University Hospital, Department of Internal Medicine, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[c] University College London, Gower Street, London, UK
[d] Gonçalo Moniz Research Center, FIOCRUZ, Salvador, BA, Brazil

SUMMARY

Background: The existence of ectosome-like microvesicles released by neutrophils was proposed a few decades ago. Other studies revealed that the innate immune response during mycobacterial infection is accompanied by an intense migration of neutrophils to the site of infection, which may be important during the acute phase of tuberculosis. We found that the ectosomes derived from infected neutrophils are biologically active and can influence the survival of *Mycobacterium tuberculosis* within macrophages.
Methods: Mycobacteria were cultured on supplemented Middlebrook-7H9 broth. All strains were grown to the exponential phase and quantitated by serial dilution. Human neutrophils and macrophages were infected with mycobacteria. Ectosomes from neutrophils were isolated post-infection and characterized by transmission electron microscopy and flow cytometry. To determine whether these microvesicles influenced mycobactericidal activity, mycobacteria-infected macrophages were treated with isolated ectosomes.
Results: Ectosomes were released from neutrophils infected with mycobacteria. These ectosomes were derived from neutrophil plasma membrane and a small proportion stained with PKH26. These microvesicles, when incubated with infected macrophages, influenced antimycobacterial activity.
Conclusions: This is the first study to demonstrate that ectosomes that are shed from infected neutrophils influence mycobactericidal activity in macrophages *in vitro*, suggesting that these microvesicles have biological significance. Nevertheless, major gaps in our knowledge of microvesicle biology remain.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Following infection with *Mycobacterium tuberculosis* (MTB), most immunocompetent adults are able to develop an immune response that controls mycobacterial proliferation. Although these individuals continue to harbor small numbers of viable mycobacteria, clinical signs of tuberculosis (TB) do not develop.[1] Pathogenic mycobacteria survive and proliferate within mononuclear phagocytes, cells that are normally capable of eliminating most internalized organisms.[2] The ability of the host to mount an effective immune and inflammatory response that results in increased mycobactericidal activity in macrophages is clearly important for controlling MTB infection. However, it is largely accepted that the optimal anti-MTB response should also restrain the inflammatory process, as excessive inflammation may lead to increased tissue damage and will contribute to TB active disease development.[3] Considerable evidence indicates that antigen-specific T-cells, activated macrophages, dendritic cells and cytotoxic T-cells are essential for containing MTB infection. Other immune and non-immune cells may also play a significant role during the host response against mycobacterial infections.[4,5] Several studies, both experimental and clinical, have shown that acute pulmonary tuberculosis is accompanied by an influx of polymorphonuclear

* Corresponding author. Rua Waldemar Falcão, 121, Candeal, CEP 40296-710, Salvador, BA, Brazil. Tel.: +55 71 3176 2232; +55 71 8803 5040 (mobile); fax: +55 71 3176 2327.
E-mail addresses: tonya.duarte@gmail.com (T.A. Duarte), adutra@pqvisitante.bahia.fiocruz.br (A.A. Noronha-Dutra), joilda_nery@yahoo.com.br (J.S. Nery), sbrmicroimuno@yahoo.com.br (S.B. Ribeiro), pitanga.tn@hotmail.com (T.N. Pitanga), jrlapa.ntg@terra.com.br (J.R. Lapa e Silva), arrudasm@gmail.com (S. Arruda), n_boechat@yahoo.com (N. Boéchat).

neutrophils (PMN).[6–11] Neutrophils migrate from the blood to the tissues and eventually ingest and kill invading pathogens, including MTB, by generating reactive oxygen intermediates together with other potent antimicrobial compounds released during the process of degranulation.[9] Human PMN have the capacity to recruit additional PMN along with other immune cells to inflammatory sites by producing and releasing chemokines and cytokines.[12–16] Several *in vitro* studies suggest that the impaired ability of PMN from TB patients to generate an effective oxidative burst may contribute to the establishment and spread of MTB infection.[9,17,18] Interestingly, activated PMN release microvesicles directly from the cell surface membrane.[19] Several different names have been used to describe these small vesicles, including microvesicles, microparticles and ectosomes, probably because the mechanism involved in their release is not known in detail.[20–24] These neutrophil-derived ectosomes are generated either spontaneously or in response to various stimuli,[20–22,24,25] and they can differ at the molecular level, depending on the trigger stimuli.[26] Importantly, the ectosomes are not just by-products of cell activation because they have been shown to have functional activity and can modulate other cells.[20,27–29] Ectosomes were recently shown to only be secreted by living cells and they are clearly distinct from the microvesicles produced by apoptotic cells.[27] In addition to their generation kinetics, ectosomes and apoptotic bodies differ in composition and size, with apoptotic bodies being many times larger in diameter and volume.[19,23] Several studies suggest that the shedding of ectosomes is an important mechanism by which PMN discharge active molecules present in their plasma membranes.[11,23,26,30] This phenomenon may result in decreased cell surface signaling activity.[31] During the early stages of inflammation, the ectosomes shed by neutrophils stimulate the release of anti-inflammatory molecules such as transforming growth factor (TGF)-β and interleukin (IL)-10 from macrophages while reducing the production of tumor necrosis factor (TNF)-α and IL-8.[11,27]

The remarkable influx and continued presence of both activated phagocyte types in the lungs during MTB infection[32,33] indicate that PMN might interact with macrophages, thereby interfering with inflammatory and antimycobacterial activity. In the present study, we demonstrate that *in vitro* MTB infection of human PMN induced the generation of PMN ectosomes. We also describe the morphological features of these ectosomes with transmission electron microscopy imaging and investigate their effect on the antibacterial activity of human macrophages against virulent MTB.

2. Materials and methods

2.1. M. tuberculosis complex strains

M. tuberculosis (H37Rv − American Type Culture Collection − ATCC 25618, New York, NY, USA) and *Mycobacterium bovis* (ATCC 19274, Rockville, MD, USA) strains were kindly provided by the National Institute of Quality Health Control (INCQS-FIOCRUZ, RJ, BR). Bacteria were rehydrated in Middlebrook-7H9 broth (Difco, MI, USA) supplemented with 10% albumin dextrose concentrate (ADC − BD BBL − Sparks, MD, USA) and maintained at 37 °C in an aerobic atmosphere for 15 days. After incubation, homogeneous cell suspensions were obtained by mild sonication using a Vibra-Cell Ultrasonic Processor (Sonics & Materials Inc., CT, USA) to disaggregate the clumps. The total number of mycobacteria in the suspension was ascertained by enumeration of colony forming units (CFU) in Middlebrook-7H10 broth (Difco, MI, USA) supplemented with 10% oleic acid albumin dextrose concentrate (OADC − BD BBL − Sparks, MD, USA) maintained at 37 °C in an aerobic atmosphere for 45 days. All of the stocks were quantified by serial dilution followed by CFU enumeration. Aliquots were stored in 20% glycerol at −80 °C until use.

2.2. Purification of PMN

Heparinized blood was obtained from healthy human volunteers. Approximately 20 mL of venous blood was gently mixed with 3% Dextran T500 (Sigma, MO, USA) and left for 30 min for erythrocyte sedimentation. The leukocyte-rich supernatant was aspirated and centrifuged at 200 g for 10 min at 24 °C. The pellet was resuspended in 12 mL of Hanks balanced salt solution (HBSS) in the absence Ca^{2+}/Mg^{2+}. This suspension was layered over 6 mL of Histopaque-1077 (Sigma, MO, USA) and centrifuged at 350 g for 20 min at 24 °C. After removal of the supernatant, PMN were recovered from the bottom of the tube. The pellet was resuspended in 2 mL of PBS and 5 mL of ultra-pure water was added to hypotonic lyses residual erythrocytes. After 1 min, isotonicity was restored by adding 2 mL of 5% NaCl. The cells were then centrifuged at 350 g for 10 min at 4 °C and resuspended in 10 mL of HBSS without Ca^{2+}/Mg^{2+}. The number of PMN was calculated by counting in a Neubauer chamber. PMN (10^6 cells/mL) were then diluted in RPMI-1640 medium (LGC, Biotechnology, SP, BR) supplemented with 10% heat-inactivated fetal calf serum − FCS (LGC, Biotechnology, SP, BR), 2 mM L-glutamine and 2 g/L sodium bicarbonate. PMN purity was determined on the basis of nuclear morphology using light microscopy and the level of viability was 95% at the time of stimulation, as determined by Trypan Blue exclusion technique (Gibco, NY, USA).

2.3. Isolation of ectosomes from infected PMN

For stimulation with mycobacteria, PMN were diluted at 10^7 cell/mL in previously warmed RPMI-1640, exposed to *M. tuberculosis* or *M. bovis* (10^7 bacilli/ml) and incubated for 2 h at 37 °C in 5% CO_2. In all cases, the supernatants were collected and centrifuged twice at 4000 g for 10 min prior to further ultracentrifugation at 100,000 g for 1 h at 4 °C. Ectosome pellets were resuspended in PBS and stored in aliquots at −80 °C prior to use.

2.4. Ultrastructural analysis of ectosomes by transmission electron microscopy (TEM)

Ectosomes isolated as described above were resuspended and fixed in a 0.1 mM cacodylate buffer containing 2% glutaraldehyde. They were then post-fixed for 1 h at room temperature with 1% osmium tetroxide in 0.1 mM cacodylate buffer. Next, they were concentrated in a 2% agar in cacodylate buffer. The samples were dehydrated in a graded series of acetone solutions and embedded in Polybed resin. To visualize the ectosomes, thin sections were sequentially stained with 2% uranyl acetate in methanol and lead citrate and examined with a Zeiss EM 109 transmission electron microscope at 80 Kv (München, Germany). Ectosomes were defined as round-shaped membrane vesicles, homogenous in size and not exceeding 300 nm in diameter.[22,30] Membrane debris was defined as heterogeneous membrane fragments of variable shapes and sizes.

2.5. Fluorescence-activated cell sorting of ectosomes and apoptotic cells

The fluorescence-activated cell sorting (FACS) analytical conditions were standardized, e.g. settings of the flow cytometry and quality of the reagents. A first series of experiments using standard silica beads of 1000 nm diameter (Corpuscular Inc., NY, USA) were run to calibrate the size and size distribution of PMN-derived ectosomes. The resulting data served as an internal control for all our ectosome-related FACS experiments. The forward scatter (FSC) and side scatter (SSC) parameters were plotted on logarithmic

scales to appropriately cover a wide size range. Microparticles presenting a diameter between 50 and 300 nm were considered as ectosomes.

To label membranes of isolated ectosomes for FACS analysis, a fluorescent cell linker dye (PKH26, Sigma, MO, USA) was used, following the labeling procedure provided by the manufacturer. The excitation and emission peaks for PKH26 are 551 nm and 567 nm, respectively. Activated neutrophils were briefly washed twice with PBS and resuspended at a concentration of 2×10^7 cells/mL in diluent C. The suspension was mixed with 1 mL of diluent C/dye solution (dye diluted 1/200) for 2–5 min at room temperature with gentle shaking. The staining reaction was stopped by adding an equal volume of FCS for 1 min at 22 °C. The sample was diluted with an equal volume of RPMI-1640 and pelleted by centrifugation for 10 min at 400 g to remove the staining solution. Ectosomes were obtained from PKH26-stained PMN by a two-step procedure involving an initial centrifugation at 4000 g for 10 min to remove cells, followed by ultracentrifugation of the supernatant at 100,000 g for 1 h at 4 °C. Unstained neutrophil-released ectosomes were analyzed under the same conditions for establishment of true negative controls. All reagents and solutions used for the ectosome analysis were sterile and filtered (0.2 μm filter).

The samples were analyzed with a FACSort Cell Sorter flow cytometer. The FSC and fluorescence channel (FL-2) were set on logarithmic scales. An acquisition threshold was set based on FSC to reduce the background signal.

Apoptotic cells were quantified by staining with annexin V and propidium iodide following the manufacturer's instructions (annexin V-FITC apoptosis detection kit – Sigma, MO, USA). Briefly, the cells were washed with binding buffer and stained with annexin V and propidium iodide for 10 min at room temperature and protected from light. FACS analysis was conducted using a FACSort Cell Sorter flow cytometer (BD Bioscience, CA, USA).

2.6. Isolation and culture of human monocyte-derived macrophages (HMDM)

Peripheral blood mononuclear cells were isolated from the buffy coat of healthy donors (provided, with permission, by the Fundação de Hematologia e Hemoterapia da Bahia – HEMOBA) by differential centrifugation, first over Histopaque-1077 and then on Percoll (LGC Biotechnology, SP, BR) gradients. The monocyte fraction was plated in 24-well plates (Tissue Culture Plate, 24 well, Becton Dickinson Labware, NJ, USA) at 10^6 cells/1000 μL/well containing RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 2 g/L sodium bicarbonate and antibiotics (100 UL/mL penicillin and 100 μL/mL streptomycin). Monocyte viability was verified by Trypan Blue exclusion technique. Cell viability at this point was greater than 95% in all experiments. Adherent cells were incubated at 37 °C, 5% CO_2 for 10 days prior to infection with mycobacteria. After 10 days in culture, monocyte morphology was analyzed by light microscopy under $400\times$ magnification (Nikon Eclipse TS 100, TYO, JP) and the cells became larger, more granular and more clustered, forming small colonies. These features were consistent with the maturation of monocytes to macrophages after 10 days of culture.

2.7. Infection of human macrophages with M. tuberculosis

Cryogenic vials containing MTB suspension were defrosted at 4 °C for 18 h, followed by reactivation in Middlebrook-7H9 broth supplemented 10% ADC for 4 h at 37 °C in an aerobic atmosphere. To infect macrophages, culture medium was removed and 100 μL of the mycobacterial suspension were added (10^6 mycobacteria/well), followed by the addition of RPMI-1640 medium supplemented

with 10% heat-inactivated FCS, 2 mM L-glutamine, 2 g/L sodium bicarbonate and antibiotics (100 UL/mL penicillin and 100 μL/mL streptomycin) to a final volume of 1000 μL/well. All macrophages were infected at a multiplicity of infection (MOI) of 1:1 in triplicate with M. tuberculosis (H37Rv). After 2 h, the cells were gently washed with warmed medium to remove extracellular bacilli. The 24-well plates containing the infected macrophages were then incubated at 37 °C and 5% CO_2 for 96 h, after which CFU counts were performed. For some experiments, wells were also treated with a suspension containing PMN-derived ectosomes. On the fourth day of infection, the medium was removed from each well and the bacilli were used immediately for enumeration.

2.8. Ectosome vesicle treatment of human macrophages infected with M. tuberculosis

HMDM were obtained, cultured in 24-well plates and infected with MTB, as described above. One hour after infection, HMDM were treated with purified ectosomes induced by MTB infection of human PMN. At two time points, 0 and 96 h post-infection, HMDM were permeabilized and the colony forming units of mycobacteria present in each well were counted.

2.9. Enumeration of colony forming units (CFU)

CFU quantification was performed according to the technique previously described by Boechat et al.[2] with some modifications. Briefly, after removing 175 μL of medium from each well, cells were permeabilized with 10 μL Middlebrook-7H9 broth containing 10% saponin (Sigma, MO, USA) for 3 min. After permeabilization, 175 μL Middlebrook-7H9 broth supplemented with 10% ADC was added and the contents of each well were dispersed by vigorous pipetting. Three serial 10-fold dilutions were prepared using Middlebrook-7H9 broth and 10 μL aliquots were plated in triplicate on Petri dishes containing Middlebrook-7H10 broth supplemented with 10% OADC. The plates were incubated at 37 °C for 3 weeks before colonies were counted. Petri dishes were incubated at 37 °C in an aerobic atmosphere for 45 days. The results are expressed as CFU/well and, in all experiments, were the average of triplicate values for each experimental condition.

2.10. Statistical methods

All results are expressed as means ± standard error of the mean (SEM) unless otherwise indicated. Comparisons between groups were made by one-way analysis of variance (ANOVA), followed by a Dunnett's post-test with comparison to the untreated or control group. Analyses were performed using the GraphPad Prism 5.0 Software (San Diego, CA). Differences with $P \leq 0.05$ were considered significant.

3. Results

3.1. Morphological characterization and PKH26 labeling of ectosomes isolated from infected PMN

The ectosomes isolated from PMN 2 h after infection with MTB were clearly identified by TEM as round-shaped membrane microvesicles with varying diameters, from 50 to 200 nm (Figure 1, panels A and B). The ectosome preparations did not contain any major impurities (Figure 1, panel C).

The PMN-derived microparticle preparations were defined by FACS analysis by size <1000 nm (Figure 2, panel A). The ectosomes were localized within the R1 and R2 regions, which encompassed particles with diameters ranging from 50 to 300 nm (Figure 2,
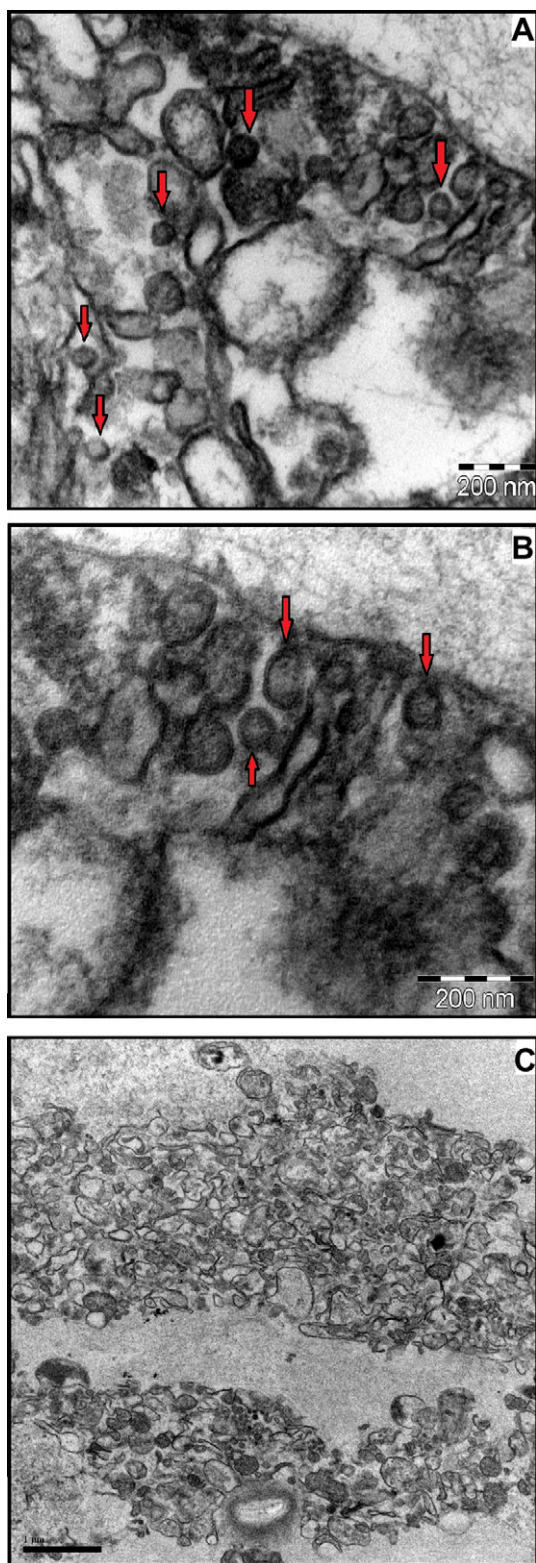
Figure 1. Transmission electron microscopy of purified ectosomes isolated from PMN. MTB-infected PMN and released microvesicles, termed ectosomes (arrows), were processed for TEM analysis 2 h after infection. All ectosomes were less than 200 nm. Scale bar: 0.2 μm (panels A and B); 1 μm (panel C).

panels B and C). Of note, only the minor microvesicle subpopulation depicted in the R1 region stained positively for PKH26. This staining pattern was observed in ectosome preparations from both infected (8.70%) and non-infected (8.33%) PMN (Figure 2, panels D and E).

3.2. Apoptosis of infected PMN

To rule out the possibility that the vesicles released from activated neutrophils were apoptotic bodies, we investigated whether *M. bovis* infection triggered early spontaneous apoptosis in PMN.

Control and *M. bovis*-infected neutrophils were stained with annexin V (AV) and propidium iodide (PI). The translocation of phosphatidylserine (PS) to the outer leaflet of the cell membrane, which can be specifically detected with AV, is one of the early events during the apoptotic process.[34] PI labels the cellular DNA in necrotic cells in which the cell membrane has been fully compromised.[34] This combination allows differentiation of early apoptotic cells (AV$^+$/PI$^-$), necrotic cells (AV$^-$/PI$^+$ or AV$^+$/PI$^+$) and viable cells (AV$^-$/PI$^-$). Following 2 h of infection, there was a significant decrease in the percentage of apoptotic and necrotic PMN, represented by the AV$^-$ and PI$^-$ events in their respective quadrants (Figure 3).

3.3. Effect of ectosomes on antimycobacterial activity of human macrophages

Macrophages can acquire neutrophil granules, impacting their antimycobacterial activity.[35] To investigate whether ectosomes released from viable PMN also interfere with the antimycobacterial activity of macrophages, MTB-infected macrophages were incubated with PMN-derived ectosomes for 96 h and, after treatment, the CFU number was determined. The addition of ectosomes reduced the antimycobacterial activity of macrophages (Figure 4). Ectosomes were derived from uninfected and MTB-infected neutrophils. Both ectosome preparations similarly inhibited the antimycobacterial activity of macrophages.

A minimal CFU number was measured when the experiments were conducted only with MTB and ectosomes, indicating that few if any bacilli were present in the ectosome preparation released from MTB-infected neutrophils. These data indicate that ectosomes provide a vehicle for regulating the antimicrobial activity of macrophages infected with intracellular mycobacteria.

4. Discussion

In response to an infectious challenge, neutrophils are among the first innate immune cells to migrate from the blood to the sites of microbial entry. Protective immunity against MTB has been traditionally ascribed to T-cells and macrophages.[1] However, the influx of PMN may also play a role in the innate immune response, especially during the initial phases of MTB infection.[36] Neutrophils secrete chemokines, such as CXCL-8, IP-10, CCL-2, MIP-1α/β,[12–15] and pro-inflammatory cytokines including IFN-γ and TNF-α[15] in response to microbial pattern recognition receptor stimulation, which can attract and instruct other immune cells. Although PMN play a major role in the defense against pathogens and in inflammatory processes, Gasser and Schifferli[11] demonstrated that PMN-derived ectosomes block the inflammatory response in HMDM by inhibiting the release of TNF-α, reducing the release of IL-8 and inducing an immediate release of TGF-β1, which is a known anti-inflammatory cytokine. In contrast, other studies suggest the opposite, proposing that microvesicles released by PMN induce a pro-inflammatory effect.[37] Mesri and Altieri[37] analyzed a mixture of microvesicles released by PMN after a very long incubation time, one at which PMN undergo apoptosis or necrosis and release many intracellular fragments, which might have contained pro-inflammatory stimuli.

Using TEM, we characterized the morphology of MTB-induced PMN-derived ectosomes. They appear as ecto-organelles surrounded by membrane. The diameter of the ectosomes generated

during the first hours of MTB infection assays ranged from 20 to 100 nm, the same range previously described for ectosomes induced by N-formylmethionyl-leucyl-phenylalanine (fMLP),[23] ionomycin[30] and MTB.[38] Apoptosis produces cell fragments appearing as vesicles, called apoptotic bodies, that phagocytic cells are able to engulf and quickly remove before the contents of the cell can spill out and damage nearby cells.[39] It is noteworthy that the diameter of apoptotic bodies is greater than 400 nm,[33] which is at least four times larger than the maximal MTB-induced ectosomes observed in our experiments. While phosphatidylserine is exposed on apoptotic bodies, it is also found on the surface of ectosomes,[40]

which allows them to be rapidly and selectively engulfed by phagocytes,[41] presumably preventing inflammation.

In 2010, Gonzalez-Cano et al.[38] published the first paper on MTB-induced PMN-derived ectosomes, showing confocal microscope images of neutrophils infected with live *Escherichia coli*, *Staphylococcus aureus* and *Leishmania mexicana* and MTB. After 10 min, all pathogens induced the release of similar microvesicles, both in terms of size and morphology. Thus far, few studies have been published on ectosome characteristics.

PMN-derived ectosomes stained poorly with PKH26. As suggested by Thom et al.,[42] it is possible that the partial staining arose
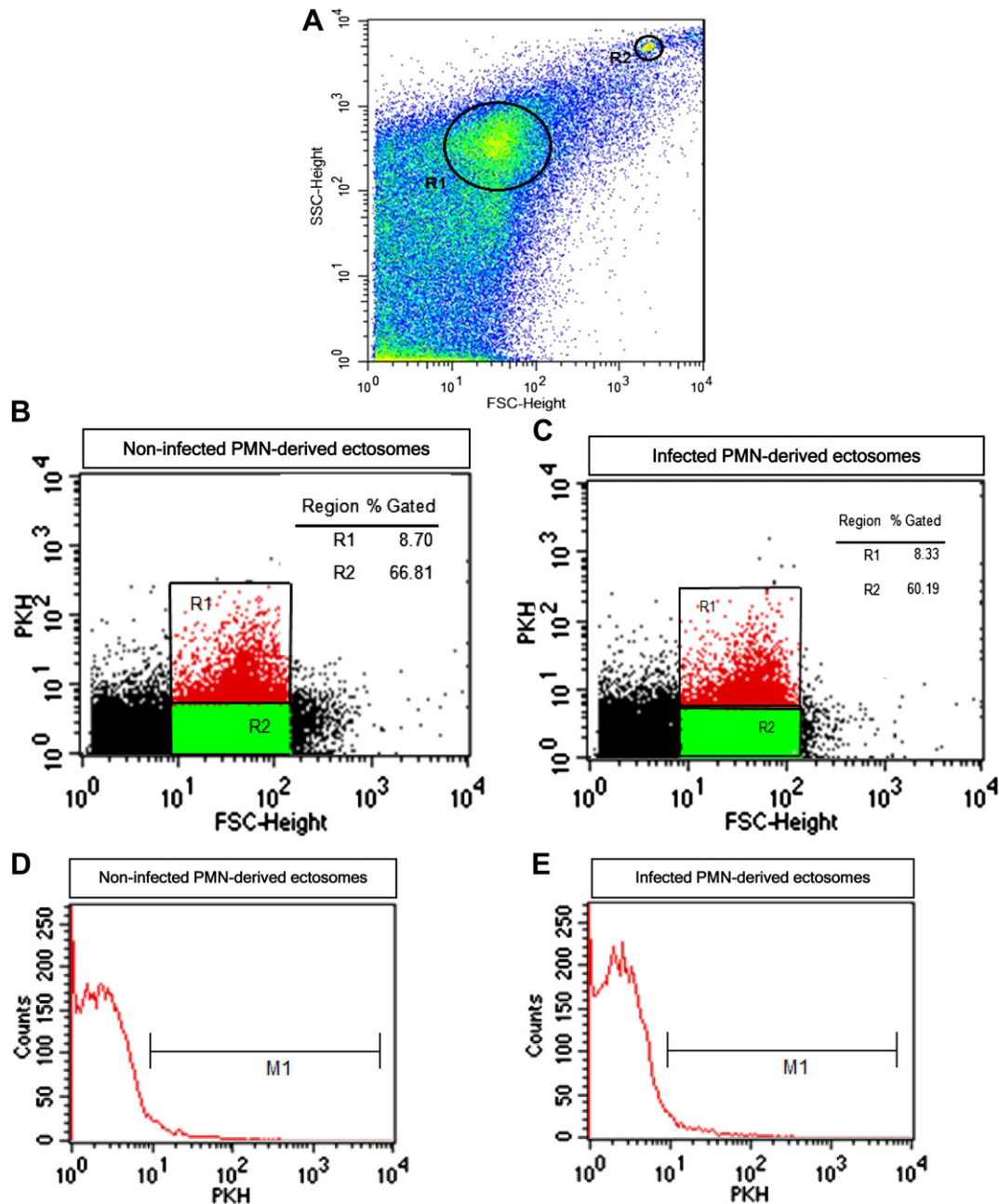
Figure 2. Purified ectosomes were tested for PKH26 positivity through flow cytometric analysis. (A) The section containing ectosomes (R1) was gated using a size < 500 nm in FSC−SSC scatter using standard beads (R2 − bead size = 1000 nm). (B−C) Panels demonstrate non-infected and infected neutrophil-derived ectosomes, respectively. Dot plots show the heterogeneity in microparticle size. The FSC and fluorescence channel (FL-2) were set on logarithmic scales. R1 and R2 regions refer to particles of approximately 200 nm in size. Ectosomes positive for PKH26 were localized within the R1 region while negative within R2 region. (D−E) Histograms demonstrate PKH26 staining in PMN-derived ectosomes (M1). Note that both non-infected and infected PMN-derived ectosomes showed partial staining for PKH26 (8.70 and 8.33%, respectively). The data are representative of three independent preparations.
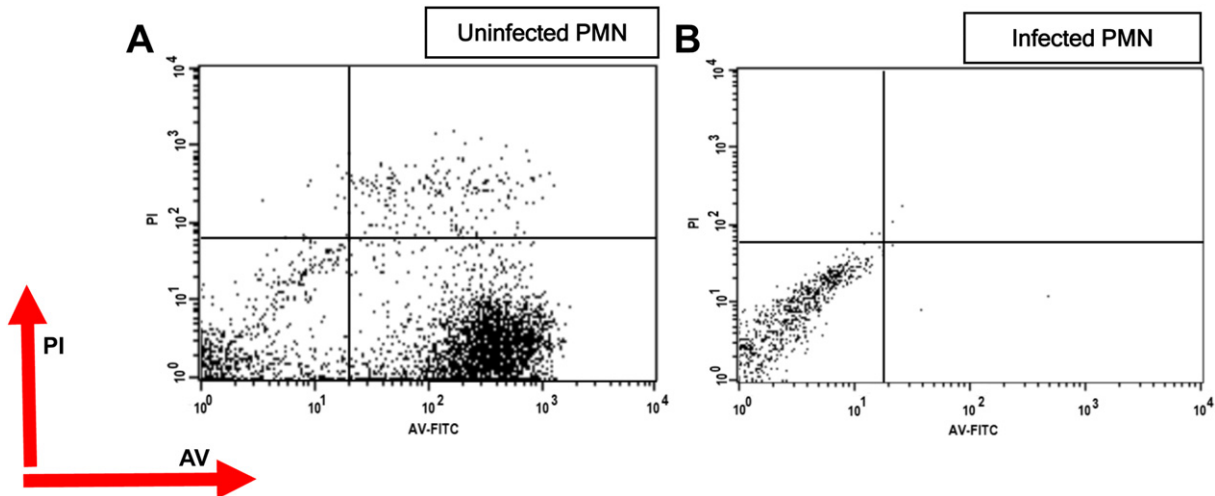
Figure 3. Flow cytometry analysis of apoptosis and necrosis in control and *M. bovis*-infected PMN. Freshly isolated PMN were infected with *M. bovis* or untreated and maintained in culture for 2 h. Cells were double stained with AV (*x* axis) and PI (*y* axis) to detect early apoptotic, necrotic or viable cells. Inhibition of PMN apoptosis and necrosis was detected in infected PMN. AV, annexin V; PI, propidium iodide.

because small vesicles (<300 nm) may exhibit a smaller variety of surface markers than parent cells.

We tested whether apoptosis was occurring in the infected PMN by staining cells with annexin V, which binds to surface phosphatidylserine and propidium iodide, which is retained in late apoptotic cells.[34] As shown in Figure 3, *M. bovis*-infected neutrophils stained negative for both annexin V and propidium iodide (1.0% AV⁻/PI⁻). However, uninfected neutrophils appeared to be undergoing apoptosis (50.7% AV⁺/PI⁻). Mycobacterial infection of PMN induces increased expression of several function-associated surface molecules and upregulates mRNA for a set of cytokines and chemokines, including IL-1α, IL-1β and MIP-1β,[34] as well as cytokine and chemokine receptors, such as IL-2Rγ, IL-10Rα and IL-6R. Furthermore, spontaneous apoptosis of activated neutrophil granulocytes is inhibited by both *M. bovis* bacillus Calmette-Guerin[43] and MTB.[44] In our experiment, MTB-induced PMN ectosomes were visualized and their secretion was enhanced upon activation but not apoptosis. These microvesicles were defined as ectosomes, in contrast to apoptotic bodies, by their morphological characteristics and by the viability of the neutrophils from which they were derived (Figure 3).

Previous studies have shown that PMN-derived ectosomes can modulate immunity and lead to an anti-inflammatory response.[11,27,45] Our study is the first to demonstrate that MTB-induced PMN-derived ectosomes interfere with the antibacterial activity of human macrophages against virulent MTB, suggesting their importance in the immune processes, particularly in the anti-TB immune response.

Major gaps remain in our knowledge of microvesicle pathophysiology and it is currently difficult to predict the biological consequences of PMN ectosome release in tissues due to their varied effects, with reported pro-inflammatory[46] and anti-inflammatory effects on monocytes and macrophages.[11,27] Mesri and Altieri[37] found that ectosomes were associated with pro-coagulant and pro-inflammatory effects. Watanabe et al.[46] described neutrophil-derived microparticles as being important pro-inflammatory agents containing platelet-activation factor (PAF), which led to the formation of platelet–leukocyte aggregates when the cells were present together. The release of PAF from stimulated PMN is a molecular mechanism with the potential to propagate inflammatory events. Trans-cellular activation by platelet and leukocyte microparticles is a well-established paradigm.[37,46]

Although ectosomes lead to mainly pro-coagulant and pro-inflammatory events in the circulation, they might be anti-inflammatory or immunosuppressive in local tissue environments. The PMN enhancement of inflammation through the release of multiple mediators and enzymes has been extensively studied, but the mechanisms responsible for controlling this reaction are less well understood. The anti-inflammatory and immunosuppressive properties of PMN-derived ectosomes are novel findings, suggesting that they are active players in the regulation of inflammation.
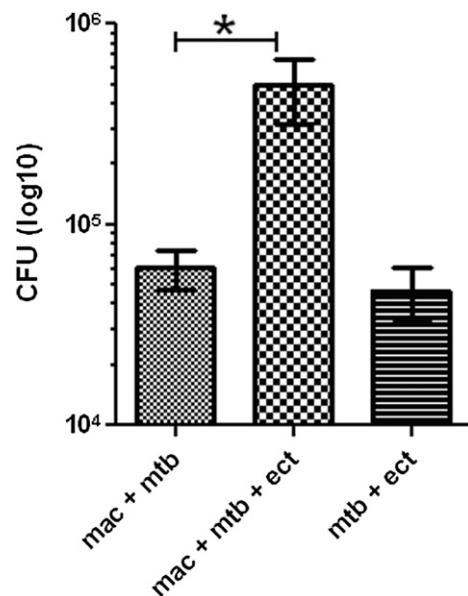
Figure 4. Influence of PMN-derived ectosomes on macrophage antimycobacterial activity. Infected PMN-derived ectosomes were added to cultures of MTB-infected macrophages. HMDM were infected with MTB at an infection ratio of 1:1 and seeded at a density of 10^6cell/well. The mean CFU number at day 0 was 10^6 for all conditions. The samples were incubated for 96 h. At 96 h post-infection, the cells were permeabilized with 10% saponin and the bacterial suspensions were collected, sonically dispersed, serially diluted and plated in triplicate on 7H10 agar plates. CFU were cultured and counted after 6 weeks. The data represent the average number of CFU ± standard error of the means (SEM) from independent experiments. The bars represent the experiments, which were performed in triplicate. *Statistically significant differences (*P* 0.0287).

Communication is a major aspect in cell biology and occurs through both direct contacts between cells and through soluble substances that react with cells. Gasser and Schifferli[11] found that PMN-derived ectosomes induced the release of TGF-β1 by HMDM in vitro and were able to block the inflammatory response of macrophages through down-regulation of IL-8, IL-10 and TNF-α secretion. Ectosome-to-cell contact was sufficient for this immunomodulatory function.

The concept that microvesicles released by some cells might influence other cells is rather new but probably represents a basic mechanism for delivering a message between cells in a highly efficient manner. The ability of PMN-derived ectosomes to down-regulate the activity of macrophages may be relevant for other inflammatory processes or infectious diseases and further investigation in this field should be pursued.

Ongoing studies to extend the knowledge of neutrophil-released ectosomes and their functional consequences may reveal novel insights into the mechanisms of cell-to-cell signaling, especially those by which ectosomes "reprogram" macrophages during acute inflammation and may also improve our understanding of the pathophysiology of MTB infection.

Acknowledgments

References

1. van Crevel R, Ottenhoff TH, van der Meer JW. Innate immunity to *Mycobacterium tuberculosis*. *Clin Microbiol Rev* 2002;**15**:294–309.
2. Boechat N, Bouchonnet F, Bonay M, Grodet A, Pelicic V, Gicquel B, et al. Culture at high density improves the ability of human macrophages to control mycobacterial growth. *J Immunol* 2001;**166**:6203–11.
3. Di Liberto D, Caccamo N, Meraviglia S, Guggino G, La Manna MP, Sireci G, et al. Tuning inflammation in tuberculosis: the role of decoy receptors. *Microbes Infect* 2009;**11**:821–7.
4. Mehta PK, Karls RK, White EH, Ades EW, Quinn FD. Entry and intracellular replication of *Mycobacterium tuberculosis* in cultured human microvascular endothelial cells. *Microb Pathog* 2006;**41**:119–24.
5. Danelishvili L, McGarvey J, Li YJ, Bermudez LE. *Mycobacterium tuberculosis* infection causes different levels of apoptosis and necrosis in human macrophages and alveolar epithelial cells. *Cell Microbiol* 2003;**5**:649–60.
6. Appelberg R. Mycobacterial infection primes T cells and macrophages for enhanced recruitment of neutrophils. *J Leukoc Biol* 1992;**51**:472–7.
7. Lasco TM, Turner OC, Cassone L, Sugawara I, Yamada H, McMurray DN, et al. Rapid accumulation of eosinophils in lung lesions in guinea pigs infected with *Mycobacterium tuberculosis*. *Infect Immun* 2004;**72**:1147–9.
8. Eruslanov EB, Lyadova IV, Kondratieva TK, Majorov KB, Scheglov IV, Orlova MO, et al. Neutrophil responses to *Mycobacterium tuberculosis* infection in genetically susceptible and resistant mice. *Infect Immun* 2005;**73**:1744–53.
9. Shalekoff S, Tiemessen CT, Gray CM, Martin DJ. Depressed phagocytosis and oxidative burst in polymorphonuclear leukocytes from individuals with pulmonary tuberculosis with or without human immunodeficiency virus type 1 infection. *Clin Diagn Lab Immunol* 1998;**5**:41–4.
10. Aleman M, Garcia A, Saab MA, De La Barrera SS, Finiasz M, Abbate E, et al. *Mycobacterium tuberculosis*-induced activation accelerates apoptosis in peripheral blood neutrophils from patients with active tuberculosis. *Am J Respir Cell Mol Biol* 2002;**27**:583–92.
11. Gasser O, Schifferli JA. Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. *Blood* 2004;**104**:2543–8.
12. Pellme S, Morgelin M, Tapper H, Mellqvist UH, Dahlgren C, Karlsson A. Localization of human neutrophil interleukin-8 (CXCL-8) to organelle(s) distinct from the classical granules and secretory vesicles. *J Leukoc Biol* 2006;**79**:564–73.
13. Luster AD. The role of chemokines in linking innate and adaptive immunity. *Curr Opin Immunol* 2002;**14**:129–35.
14. Kobayashi M, Tsuda Y, Yoshida T, Takeuchi D, Utsunomiya T, Takahashi H, et al. Bacterial sepsis and chemokines. *Curr Drug Targets* 2006;**7**:119–34.
15. Korbel DS, Schneider BE, Schaible UE. Innate immunity in tuberculosis: myths and truth. *Microbes Infect* 2008;**10**:995–1004.
16. Territo MC, Ganz T, Selsted ME, Lehrer R. Monocyte-chemotactic activity of defensins from human neutrophils. *J Clin Invest* 1989;**84**:2017–20.
17. Aleman M, Beigier-Bompadre M, Borghetti C, de la Barrera S, Abbate E, Isturiz M, et al. Activation of peripheral blood neutrophils from patients with active advanced tuberculosis. *Clin Immunol* 2001;**100**:87–95.
18. Fiorenza G, Farroni MA, Bogue C, Selenscig D, Lamas DM, Dlugovitzky D. Functional characteristics of neutrophils and mononuclear cells from tuberculosis patients stimulated in vitro with heat killed *M. tuberculosis*. *Arch Med Res* 2007;**38**:526–33.
19. Pluskota E, Woody NM, Szpak D, Ballantyne CM, Soloviev DA, Simon DI, et al. Expression, activation, and function of integrin alphaMbeta2 (Mac-1) on neutrophil-derived microparticles. *Blood* 2008;**112**:2327–35.
20. Nolan S, Dixon R, Norman K, Hellewell P, Ridger V. Nitric oxide regulates neutrophil migration through microparticle formation. *Am J Pathol* 2008;**172**:265–73.
21. Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol* 2009;**19**:43–51.
22. Pilzer D, Gasser O, Moskovich O, Schifferli JA, Fishelson Z. Emission of membrane vesicles: roles in complement resistance, immunity and cancer. *Springer Semin Immunopathol* 2005;**27**:375–87.
23. Gasser O, Hess C, Miot S, Deon C, Sanchez JC, Schifferli JA. Characterization and properties of ectosomes released by human polymorphonuclear neutrophils. *Exp Cell Res* 2003;**285**:243–57.
24. Stein JM, Luzio JP. Ectocytosis caused by sublytic autologous complement attack on human neutrophils. The sorting of endogenous plasma-membrane proteins and lipids into shed vesicles. *Biochem J* 1991;**274**:381–6.
25. Pfeffer SR. Two rabs for exosome release. *Nat Cell Biol* 2010;**12**:3–4.
26. Sadallah S, Eken C, Schifferli JA. Ectosomes as immunomodulators of inflammation and immunity. *Clin Exp Immunol* 2011;**163**:26–32.
27. Eken C, Gasser O, Zenhaeusern G, Oehri I, Hess C, Schifferli JA. Polymorphonuclear neutrophil-derived ectosomes interfere with the maturation of monocyte-derived dendritic cells. *J Immunol* 2008;**180**:817–24.
28. Distler JH, Huber LC, Hueber AJ, Reich 3rd CF, Gay S, Distler O, et al. The release of microparticles by apoptotic cells and their effects on macrophages. *Apoptosis* 2005;**10**:731–41.
29. Andrews RK, Berndt MC. Microparticles facilitate neutrophil/platelet crosstalk. *Blood* 2008;**112**:2174–5.
30. Hess C, Sadallah S, Hefti A, Landmann R, Schifferli JA. Ectosomes released by human neutrophils are specialized functional units. *J Immunol* 1999;**163**:4564–73.
31. Qu Y, Dubyak GR. P2X7 receptors regulate multiple types of membrane trafficking responses and non-classical secretion pathways. *Purinergic Signal* 2009;**5**:163–73.
32. Abdul-Majid KB, Ly LH, Converse PJ, Geiman DE, McMurray DN, Bishai WR. Altered cellular infiltration and cytokine levels during early *Mycobacterium tuberculosis* sigC mutant infection are associated with late-stage disease attenuation and milder immunopathology in mice. *BMC Microbiol* 2008;**8**:151.
33. Ozaki T, Nakahira S, Tani K, Ogushi F, Yasuoka S, Ogura T. Differential cell analysis in bronchoalveolar lavage fluid from pulmonary lesions of patients with tuberculosis. *Chest* 1992;**102**:54–9.
34. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* 1995;**184**:39–51.
35. Tan BH, Meinken C, Bastian M, Bruns H, Legaspi A, Ochoa MT, et al. Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens. *J Immunol* 2006;**177**:1864–71.
36. Eum SY, Kong JH, Hong MS, Lee YJ, Kim JH, Hwang SH, et al. Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. *Chest* 2010;**137**:122–8.
37. Mesri M, Altieri DC. Endothelial cell activation by leukocyte microparticles. *J Immunol* 1998;**161**:4382–7.
38. Gonzalez-Cano P, Mondragon-Flores R, Sanchez-Torres LE, Gonzalez-Pozos S, Silva-Miranda M, Monroy-Ostria A, et al. *Mycobacterium tuberculosis* H37Rv induces ectosome release in human polymorphonuclear neutrophils. *Tuberculosis* 2010;**90**:125–34.
39. Savill J. Recognition and phagocytosis of cells undergoing apoptosis. *Br Med Bull* 1997;**53**:491–508.
40. Dalli J, Norling LV, Renshaw D, Cooper D, Leung KY, Perretti M. Annexin 1 mediates the rapid anti-inflammatory effects of neutrophil-derived microparticles. *Blood* 2008;**112**:2512–9.

41. Wu Y, Tibrewal N, Birge RB. Phosphatidylserine recognition by phagocytes: a view to a kill. *Trends Cell Biol* 2006;**16**:189—97.
42. Thom SR, Yang M, Bhopale VM, Huang S, Milovanova TN. Microparticles initiate decompression-induced neutrophil activation and subsequent vascular injuries. *J Appl Physiol* 2010;**110**:340—51.
43. Suttmann H, Lehan N, Bohle A, Brandau S. Stimulation of neutrophil granulocytes with *Mycobacterium bovis* bacillus Calmette-Guerin induces changes in phenotype and gene expression and inhibits spontaneous apoptosis. *Infect Immun* 2003;**71**:4647—56.
44. Blomgran R, Desvignes L, Briken V, Ernest JD. *Mycobacterium tuberculosis* inhibits neutrophil apoptosis, leading to delayed activation of naïve CD4 T cell. *Cell Host Microbe* 2012;**11**:81—90.
45. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune response. *Nat Rev Immunol* 2009;**9**:581—93.
46. Watanabe JJ, Marathe GK, Neilsen PO, Weyrich AS, Harrison KA, Murphy RC, et al. Endotoxins stimulate neutrophil adhesion followed by synthesis and release of platelet-activating factor in microparticles. *J Biol Chem* 2003;**278**:33161—8.